

**MECHANISMS OF PROLACTIN AND GROWTH HORMONE GENE REGULATION  
IN NORMAL AND ABNORMAL PITUITARY TISSUE**

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## SYNOPSIS

The work described in this thesis involves a series of studies on the hormonal and intracellular regulation of the expression of the prolactin (PRL) and growth hormone (GH) genes. The studies are based on both rat pituitary tissue and human pituitary adenoma tissue, and the regulation of both rat and human genes is discussed.

In initial studies cDNA/mRNA hybridization assays were used to assess the regulation of messenger RNA production, first in primary cell cultures of rat pituitary tissue, and then in a series of GH or PRL-secreting human pituitary adenomas. The effects of dopamine and dopaminergic drugs, somatostatin, hypothalamic releasing hormones and intracellular signalling systems were variable among different human tumours, but in general hPRL and hGH mRNA levels in tumour cells were less responsive to inhibition than rPRL or rGH mRNA levels in normal rat pituitary tissue.

Secondly, the potential mechanisms for PRL gene regulation by intracellular signals were studied using the clonal rat pituitary GH<sub>3</sub> cell line. Transfection analysis was used to assess the role of the promoter region of the rat PRL gene in calmodulin-dependent regulation of transcription: a defined region of proximal 5'-flanking DNA conferred marked calcium/calmodulin responsiveness onto a bacterial reporter gene. The role of nuclear protein binding to this region of the gene was studied using gel mobility shift assays, and this technique was used to study both the possible mechanisms for the calcium/calmodulin regulation of nuclear protein binding to DNA in PRL gene expression. The results showed that nuclear protein binding to DNA is affected by calmodulin antagonists, and suggest that the mechanisms for intracellular regulation of the PRL gene overlap with those of its tissue-specific expression.

Finally, preliminary attempts were made to apply the transfection and gel shift techniques to human pituitary tumour tissue, and showed that such techniques may indeed be applicable even to small quantities of pituitary tumour tissue, and may throw some light on possible pathogenetic mechanisms for pituitary adenoma formation.



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*What an incredible number of layers!*  
*Don't we get to the heart of it soon?*  
*No, I'm damned if we do. Right down to the centre*  
*there's nothing but layers - smaller and smaller...*  
*Nature is witty!*

Ibsen, Peer Gynt, Act 5

## ABBREVIATIONS

AP-1, AP-2	Activator proteins 1 & 2
ATP	adenosine triphosphate
BSA	bovine serum albumin
cAMP	cyclic adenosine 3'5'-monophosphate
CaM	calmodulin
CAT	chloramphenicol acetyl transferase
cDNA	complementary DNA
C/EBP	CCAAT enhancer binding protein
cpm	counts per minute
CRE	cAMP response element
CREB	CRE binding protein
CS	chorionic somatomammotrophin
CT	computerised tomography
dCTP	deoxycytidine triphosphate
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I
dNTP	deoxynucleotide triphosphate
dpm	disintegrations per minute
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
EGF	epidermal growth factor
ERE	oestrogen response element
FGF	fibroblast growth factor (bFGF = basic FGF)
GABA	$\gamma$ -amino butyric acid
GH	growth hormone
GH <sub>3</sub>	rat pituitary cell line; GH line subclone
GI	guanidine isothiocyanate
GRE	glucocorticoid response element
GRF	growth hormone releasing factor
hGH	human growth hormone
hPRL	human prolactin

IC <sub>50</sub>	median inhibitory concentration
IGF-I	insulin-like growth factor I
kD	kiloDaltons
mRNA	messenger ribonucleic acid
PBS	phosphate buffered saline
Pit-1/GHF-1	pituitary-specific (growth hormone) factor-1
PMSF	phenylmethyl sulphonyl fluoride
PRL	prolactin
r	rat
rGH	rat growth hormone
rPRL	rat prolactin
RNA	ribonucleic acid
RSV	Rous sarcoma virus
SEM	standard error of the mean
SSC	standard saline citrate (150mM NaCl; 15mM trisodium citrate)
T <sub>3</sub>	tri-iodothyronine
TLC	thin layer chromatography
TPA	12-O-tetradecanoyl phorbol-13-acetate
TRH	thyrotrophin-releasing hormone
TSH	thyroid stimulating hormone
VIP	vasoactive intestinal peptide
W7	N-(6-aminohexyl)-1-naphthalene sulphonamide
W8	5-iodo-1-C8 (iodo-W7 derivative)

## CONTENTS

	Page
<b>CHAPTER 1</b>	
<b>PROLACTIN AND GROWTH HORMONE GENE REGULATION</b>	
1.1 INTRODUCTION	1
1.2 THE PROLACTIN AND GROWTH HORMONE GENE FAMILY	2
1.2.1 Gene structure and relationships	2
1.2.2 Ontogeny	6
1.3 TRANSCRIPTIONAL REGULATION	7
1.4 TISSUE-SPECIFIC EXPRESSION	10
1.4.1 Prolactin	11
1.4.1.1 Cis elements	11
1.4.1.2 Cis/trans interactions	12
1.4.1.3 Human prolactin gene	14
1.4.2 Growth hormone	16
1.4.2.1 Cis elements	16
1.4.2.2 Cis/trans interactions	17
1.4.2.3 Human growth hormone gene	19
1.4.3 Summary: function and nature of Pit-1/GHF-1	20
1.5 HORMONAL AND INTRACELLULAR REGULATION	24
1.5.1 Prolactin	25
1.5.1.1 Nuclear hormone receptors	25
1.5.1.2 Peptide and dopamine regulation	28
1.5.1.3 Intracellular messenger pathways	30
1.5.2 Growth hormone	33
1.5.2.1 Thyroid hormone	33
1.5.2.2 Glucocorticoids	35
1.5.2.3 Peptide hormone regulation and intracellular messenger pathways	36
1.5.2.4 Human growth hormone gene	37
1.5.3 Summary: intracellular control of transcription	38
1.6 CONCLUSIONS	40
 <b>CHAPTER 2</b>	
<b>DOPAMINERGIC REGULATION OF RAT PROLACTIN mRNA ACCUMULATION</b>	
2.1 INTRODUCTION	42
2.2 METHODS	45

2.3	RESULTS	46
2.3.1	Dose-dependent inhibition of PRL release and mRNA levels	46
2.3.2	Pooled data for 100nM doses of CV 205-502 and CQP 201-403	46
2.4	DISCUSSION	51
<b>CHAPTER 3</b>	<b>AGGRESSIVE PROLACTINOMA: CLINICAL AND CELL CULTURE STUDIES</b>	
3.1	INTRODUCTION	53
3.2	CASE REPORT	54
3.3	CELL CULTURE STUDIES	60
3.3.1	Methods	60
3.3.2	Results	61
3.4	DISCUSSION	64
<b>CHAPTER 4</b>	<b>GROWTH HORMONE mRNA REGULATION IN HUMAN SOMATOTROPH TUMOURS</b>	
4.1	INTRODUCTION	67
4.2	MATERIALS AND METHODS	68
4.2.1	Cell culture	68
4.2.2	Materials and radioimmunoassays	70
4.2.3	mRNA measurements	71
4.3	RESULTS	71
4.4	DISCUSSION	78
<b>CHAPTER 5</b>	<b>CALMODULIN-DEPENDENCE OF PROLACTIN GENE EXPRESSION: HYBRIDIZATION AND TRANSFECTION STUDIES</b>	
5.1	INTRODUCTION	83
5.2	MATERIALS AND METHODS	84
5.2.1	GH <sub>3</sub> cell culture and radioimmunoassays	84
5.2.2	Hybridization studies: mRNA measurements	85
5.2.3	Transient transfection studies	85
5.2.4	Assay for CAT activity	86
5.2.5	Materials	87
5.3	RESULTS	87
5.3.1	Hybridization experiments	87
5.3.2	Transfection experiments	87
5.4	DISCUSSION	96

<b>CHAPTER 6</b>	<b>STUDIES OF DNA-PROTEIN INTERACTIONS WITH THE PROLACTIN GENE PROMOTER REGION: GEL RETARDATION ASSAYS</b>	
6.1	INTRODUCTION	100
6.2	METHODS	104
6.2.1	Nuclear protein preparation from GH <sub>3</sub> cells	104
6.2.2	Gel retardation analysis	105
6.3	RESULTS	106
6.3.1	Effect of GH <sub>3</sub> nuclear protein on PRL -172/+33 fragment	106
6.3.2	Effect of GH <sub>3</sub> nuclear protein on PRL-P3 oligonucleotide	107
6.3.3	Effect of calmodulin antagonists on nuclear protein binding	107
6.4	DISCUSSION	112
 <b>CHAPTER 7</b>	 <b>APPLICATION OF TRANSIENT TRANSFECTION AND GEL RETARDATION ASSAY TO HUMAN TISSUES: PITUITARY AND PLACENTA</b>	
7.1	INTRODUCTION	115
7.2	METHODS	116
7.2.1	Transient transfection of human prolactinoma cells	116
7.2.2	Normal bovine pituitary tissue: nuclear protein extraction	118
7.2.3	Normal human pituitary, pituitary tumour and placenta: nuclear protein extraction	119
7.2.4	Gel retardation assays	120
7.3	RESULTS	121
7.3.1	Transient transfection of prolactinoma cells	121
7.3.2	Gel retardation studies	123
7.4	DISCUSSION	129
 <b>CHAPTER 8</b>	 <b>CONCLUSIONS: MOLECULAR APPROACHES TO THE PATHOGENESIS OF PITUITARY TUMOURS</b>	
8.1	INTRODUCTION	132
8.2	GENE REGULATION AND PITUITARY ADENOMA PATHOGENESIS	132
8.2.1	Studies of mRNA regulation in pituitary tumours	133
8.2.2	Hormone gene structure and proto-oncogenes	134
8.2.3	Abnormalities in membrane proteins: application of PCR	135
8.3	CONCLUSION	136

## **APPENDIX 1                      CELL CULTURE PROCEDURES**

A1.1	Primary cell culture technique	138
A1.2	GH <sub>3</sub> cell culture technique	140

## **APPENDIX 2                      RADIOLABELLING OF DNA SEQUENCES**

A2.1	Labelling of cDNA probes by nick translation	142
A2.2	Labelling of hGH cDNA by random primer technique	144
A2.2.1	Excision of hGH cDNA insert	144
A2.2.2	Radiolabelling of excised cDNA probe	145
A2.3	Labelling of oligonucleotides with T4 polynucleotide kinase	146

## **APPENDIX 3                      HYBRIDIZATION TECHNIQUES**

A3.1	Cytoplasmic dot hybridization assay	148
A3.1.1	Preparation of cytoplasmic samples	148
A3.1.2	Hybridization assay	148
A3.2	Northern blot analysis	151
A3.2.1	RNA preparation: guanidine isothiocyanate/caesium chloride	151
A3.2.2	Agarose gel electrophoresis and Northern blotting	153

## **APPENDIX 4                      PROLACTIN AND GROWTH HORMONE RADIOIMMUNOASSAYS**

A4.1	Prolactin radioimmunoassay	156
A4.1.1	Buffers and materials	156
A4.1.2	Assay procedure	157
A4.2	Growth hormone radioimmunoassay	158
A4.2.1	Buffers and reagents	158
A4.2.2	Assay procedure	159

## **APPENDIX 5                      PROTEIN ASSAY**

A5.1	Protein assay: Lowry technique	160
A5.1.1	Materials	160
A5.1.2	Assay procedure	160

<b>REFERENCES</b>	<b>161</b>
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## CHAPTER 1

### PROLACTIN AND GROWTH HORMONE GENE REGULATION

#### 1.1 INTRODUCTION

Pituitary adenomas presenting with hypersecretion of prolactin (PRL) or growth hormone (GH) are important problems in clinical endocrinology. Despite much research the pathogenesis of human pituitary adenomas has until recently remained almost completely unknown. The past ten years have seen an enormous advance in our knowledge of the structure and expression of the PRL and GH genes, and also of some of the important proteins which are involved in their transcriptional regulation. The fruits of this new understanding are just beginning to be applied more directly to the study of human disease. Major advances have recently been made in knowledge of basic cellular control mechanisms in normal and neoplastic pituitary tissue, and these may well influence future therapeutic strategies for diseases of the pituitary.

This chapter will present a brief description of the PRL-GH gene family, with a review of our present understanding of tissue-specificity and hormonal regulation in the control of gene transcription. This general introduction will form the background to an attempt in this thesis to apply several recently developed molecular biological techniques to human pituitary tumour tissue. The use of hybridization analysis will first be illustrated in studies of PRL mRNA regulation in normal rat pituitary tissue (Chapter 2), and then applied to studies of PRL- and GH-secreting human pituitary adenomas which suggest abnormal hormonal regulation of hPRL and hGH mRNA accumulation (Chapters 3 and 4). Then studies of gene

promoter function and DNA-protein interactions will be described with applications of the techniques of transient transfection analysis and gel retardation analysis to a study of calcium/calmodulin regulation of rat PRL gene promoter function (Chapters 5 and 6). Preliminary applications of these techniques to pituitary tumour tissue will be described with tentative evidence for possible abnormal DNA-protein interactions (Chapter 7) before a concluding assessment of our present understanding of the molecular pathogenesis of pituitary adenomas (Chapter 8).

## 1.2 THE PROLACTIN AND GROWTH HORMONE GENE FAMILY

### 1.2.1 Gene structure and relationships

The genes coding for PRL and GH are related and form part of a family which also comprises chorionic somatomammotrophin (CS, or placental lactogen, PL; reviewed in Miller & Eberhardt, 1983). In the rat there is a single GH gene (Barta et al, 1981), a single rPRL gene (Cooke & Baxter, 1982) and several rCS/PL genes (Duckworth et al, 1986). In man, while the single hPRL gene (Cooke et al, 1981; Truong et al, 1984) is located on chromosome 6 (Owerbach et al, 1981), there is a cluster of related hGH and hCS/hPL genes (Martial et al, 1979; Seeburg, 1982) grouped together on chromosome 17 (Owerbach et al, 1980; George et al, 1981; Kidd & Saunders, 1982; Hirt et al, 1987), as shown in Figure 1.1. The single hGH-N gene is expressed primarily in the pituitary and alternative splicings of the nuclear pre-mRNA generate multiple mature cytoplasmic mRNAs, among which one encodes the 20kd variant protein (which constitutes 5-10% of pituitary GH), and another would code for a novel 17.5kd protein (Lecomte et al, 1987). The variant GH gene, hGH-V, was first shown to be functional (that is, not a pseudogene) by transfection studies using a viral promoter to

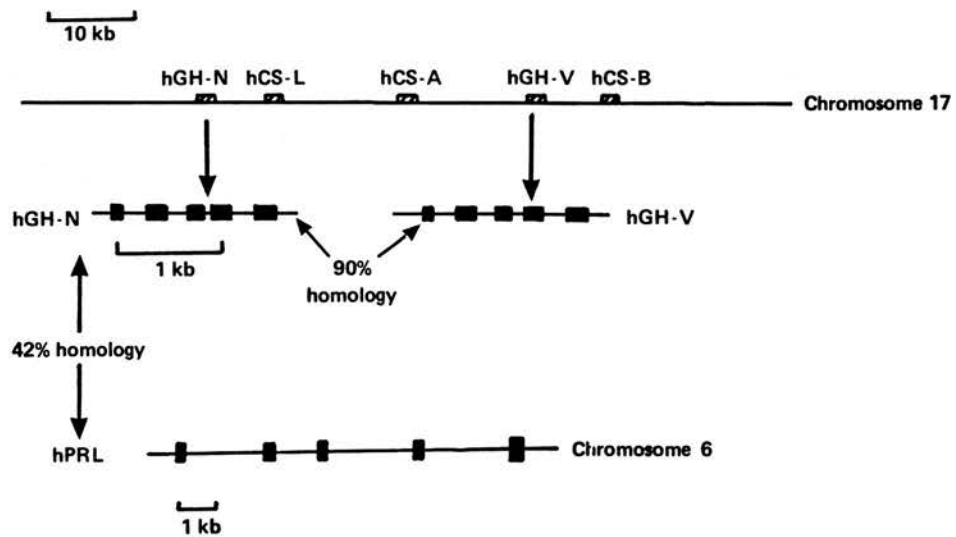


Figure 1.1

Arrangement of human GH/CS gene cluster and the structure of human GH-N, GH-V and PRL genes, with exons shown as black boxes and respective scales shown in kilobases (kb).

direct its expression in monkey kidney cells *in vitro* (Pavlakakis et al, 1981); however the endogenous human hGH-V mRNA was only recently found in placental tissue and in very small amounts in a pituitary adenoma (Frankenne et al, 1987). The structure of hGH-V mRNA has recently been determined from a human placental cDNA library, and two different mRNAs appear to be generated by alternative splicing of the gene, giving rise to two distinct proteins (Cooke et al, 1988). The hCS-L gene is thought to be a non-functional pseudogene, but hCS-A and hCS-B are expressed in syncytiotrophoblast tissue (McWilliams & Boime, 1980).

Among the human genes there is considerable coding sequence homology, up to 92% between hGH and hCS (Cooke et al, 1980; Cooke et al, 1981), and 42% between the hPRL and hGH genes (Martial et al, 1979; Seeburg, 1982; Truong et al, 1984). Indeed between species there is significant homology of coding sequences, 73% between human and rat PRL and 75% between human and rat GH (Cooke et al, 1981). It has been calculated from data on the conservation of coding sequences and splicing sites that the PRL and GH genes derived from a single primordial gene and began to diverge about 400 million years ago, the period when fish and quadripeds started their evolutionary divergence (Cooke et al, 1981; Cooke & Baxter, 1982). Interestingly, the proximal regulatory 5'-flanking DNA sequences are also highly conserved between the rat, bovine and human PRL genes (Cooke et al 1982; Camper et al, 1984; Truong et al, 1984), particularly in regions thought to be important for tissue-specific gene expression.

Further members of the PRL gene family have recently been described in other tissues. Distinct from the placental lactogen genes, "proliferin" has been identified from a mouse cDNA library, and appears to be linked to cellular proliferation (Linzer & Nathans, 1984; Linzer et al, 1985; Lee &

Nathans, 1987); a placental "proliferin-related protein" gene and a third prolactin-like protein and gene have also been described (Linzer & Nathans, 1985; Colosi et al, 1988; Duckworth et al, 1988). These genes map to chromosome 13 in the mouse, along with PRL itself (Jackson-Grusby et al, 1988) and show some sequence homology with PRL, but their tissue specificity differs.

PRL itself is synthesized at low levels in non-pituitary tissue, notably in human decidual endometrial tissue during pregnancy, although its regulation differs substantially from that in the pituitary gland. For example decidual PRL secretion is unaffected by dopamine (Golander et al, 1979; Richards et al, 1982) or oestrogen (Daly et al, 1983; Rosenberg & Bhatnagar, 1984), yet is stimulated by progesterone (Riddick & Daly, 1982; Daly et al, 1983), and by insulin and insulin-like growth factor 1 (Thraill et al, 1988, 1989).

So far there is no information regarding possible hormonal regulation of decidual PRL mRNA accumulation. PRL mRNA has been detected in human decidual cells (Clements et al, 1983; Taii et al, 1984), and the human decidual PRL cDNA sequence is almost identical to that of the pituitary cDNA, confirming that there is only a single copy of the hPRL gene per haploid genome (Takahashi et al, 1984). However the decidual mRNA has a slightly different size from pituitary mRNA which is not accounted for by the length of the poly-adenylated tail (Gellersen et al, 1989); this might suggest either alternative splicing of nuclear pre-mRNA or a different transcription initiation site, possibly due to different transcription factors in decidual cells.

Finally, prolactin mRNA and protein production occurs at low levels in human IM-9 lymphocytic cells, but whether this may imply some latent

biological function remains to be seen as normal human lymphocytes have no detectable prolactin mRNA (Dimattia et al, 1988).

### 1.2.2 Ontogeny

Despite the close structural similarities between their genes, PRL and GH exhibit quite distinct developmental patterns and tissue-specific expression. PRL and GH are produced in separate cell types within the pituitary gland, lactotrophs and somatotrophs, and immunocytochemical studies suggest that they are expressed in the pituitary only late in fetal development (Gash et al, 1982). However, rat PRL and GH may be co-expressed in the same cell, and such somatomammotrophs may predominate in the late fetal pituitary, according to an immunocytochemical study (Hoeffler et al, 1985). In the rat, a comparison of immunocytochemical studies with in situ hybridization data has recently shown that PRL mRNA accumulates well before PRL can be detected by immunostaining; as PRL is secreted in fetal rats, this indicates that it is synthesized but not stored in the fetal pituitary, possibly reflecting lack of fetal hypothalamic dopamine production (Hooghe-Peters et al, 1988).

Co-production of PRL and GH has been shown in adult rat pituitary tissue both by immunocytochemical studies and also by reverse haemolytic plaque assays (Frawley et al, 1985; Nikitovich-Winer et al, 1987). The potential for co-expression of the PRL and GH genes in a single cell type has suggested that tissue-specific expression of these genes may be at least partly controlled by a single common factor (Nelson et al, 1988), and experimental support for this hypothesis will be discussed in detail in the next section.

### 1.3 TRANSCRIPTIONAL REGULATION

The regulation of gene transcription is currently considered to be exerted by the specific interaction of regulatory proteins (*trans* acting factors) with given DNA sequences (*cis* elements). Different types of *cis/trans* coupling are thought to allow both tissue-specific expression and also hormonal induction or inhibition of gene transcription. The *cis* regulatory sequences are frequently located within promoter regions, or may be part of a separate "enhancer" element that can regulate transcription at a distance, either within or outside of the structural gene, upstream or downstream from the transcription initiation site (Figure 1.2). Changes in the three-dimensional structure of DNA may allow the DNA-bound *trans* factors to interact with the transcription initiation complex (as shown schematically in Figure 1.3, and for reviews see Dynan & Tjian, 1985; Ptashne, 1986; Maniatis et al, 1987 and Ptashne, 1988). Several *trans* factors have been identified (some of which correspond to cellular proto-oncogenes), including steroid and thyroid hormone receptors (see Evans, 1988, for review) and activator proteins such as AP1 and AP2 which are thought to mediate the effects of intracellular messenger systems (Lee et al, 1987; Imagawa et al, 1987). The structure of many of these factors is now becoming understood, and indicates that they may interact, for example by dimerization, to activate gene transcription (see Mitchell & Tjian, 1989, for review).

The regulation of PRL and GH gene transcription involves two aspects: tissue-specific expression and hormonal regulation. Although these will be considered separately, the final level of transcriptional activity presumably reflects integration of both systems, and indeed it seems likely that their mechanisms overlap to a great extent. A general approach to

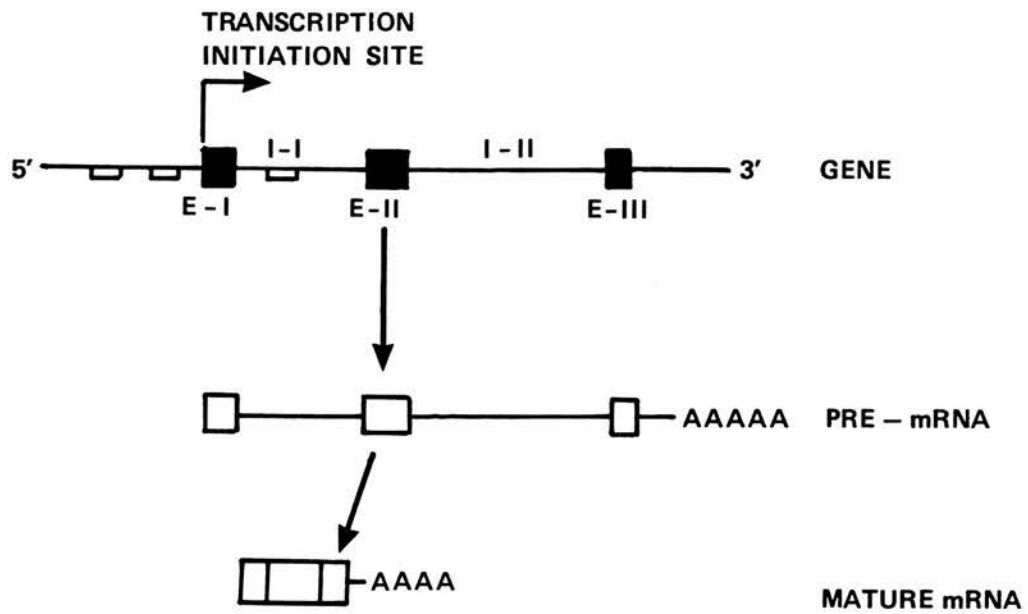


Figure 1.2

Arrangement of putative regulatory regions (open boxes) in relation to the transcription initiation site, exons (E; black boxes) and introns (I) in a model gene. The generation and splicing of polyadenylated messenger RNA are shown.



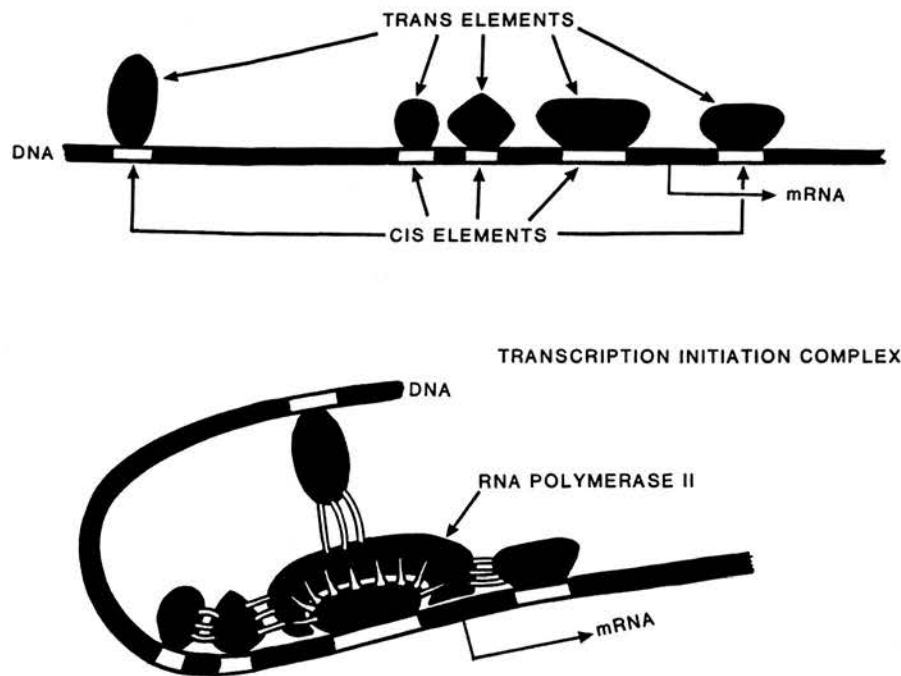


Figure 1.3

Possible interaction of *cis* and *trans* elements. Upper: *cis* elements in the gene are indicated as white areas to which *trans*-acting factors (black blobs) are bound. The transcription initiation site is shown by the right-angled arrow. Lower: possible interaction of bound *trans* factors with RNA polymerase II by looping of DNA; protein-protein interactions are suggested by links between the *trans* factors within a transcription initiation complex.

understanding these mechanisms should involve (i) the identification of *cis* elements in a given gene; (ii) precise definition of *cis/trans* interactions - that is, demonstration of protein-DNA binding and identification of *trans* acting factors involved; and (iv) purification and cloning of *trans* acting factors, and assessment of their regulation. Such an approach has been used with great success in the case of the PRL and GH genes, for example with the definition of steroid and thyroid hormone receptor structure and DNA binding characteristics, and also the identification of the pituitary tissue specific factor Pit-1/GHF-1, and these will be considered in detail next.

#### 1.4 TISSUE-SPECIFIC EXPRESSION

The PRL and GH genes are present in all cells, but are expressed only in pituitary or placental tissue; indeed the respective levels of mRNA for hGH-N and hCS may differ by as much as 10,000-fold despite considerable sequence homology, raising the intriguing question of the possible mechanisms which confer this tissue-specificity of gene expression. In the case of both PRL and GH it is noteworthy that segments of the 5'-flanking sequence are markedly conserved between species, suggesting their functional importance. The PRL and GH genes have normally been considered separately, and they will be discussed separately here before summarising a picture of our current understanding. However, the mechanisms responsible for conferring tissue specificity for PRL and GH gene expression may in fact be at least partly shared, and a common pituitary *trans*-acting factor, Pit-1/GHF-1, has recently been identified which appears to bind to the upstream elements not only of the PRL and GH genes, but also the TSH- $\beta$  subunit gene (Nelson et al, 1988). This factor has subsequently been

cloned and identified by two independent groups (Bodner et al, 1988; Ingraham et al, 1988). The hypothesis of a common tissue-specific targeting mechanism which operates *in vivo* has received further support from the recent finding that upstream elements of the GH gene can direct expression of the gene in transgenic mice to lactotrophs and thyrotrophs as well as to somatotrophs (Lira et al, 1988).

#### 1.4.1 PROLACTIN

##### 1.4.1.1 Cis elements

The technique of transfection analysis has been used to study the role of the 5'-flanking DNA sequences of the rPRL gene. This technique involves the construction of fusion genes which contain putative regulatory elements linked to reporter genes such as bacterial chloramphenicol acetyl transferase (CAT); the expression of CAT activity is therefore a function of promoter activity and can be readily measured in mammalian cell extracts. By transfecting rat pituitary GH<sub>3</sub> cells with a series of PRL-CAT fusion genes containing up to 3kb of PRL 5'-flanking DNA, Nelson et al (1986) identified two regulatory sequences whose deletion led to a drop in CAT gene transcription. A distal element located between -1831 and -1530 base pairs (bp) upstream of the transcription initiation site (or "cap site") accounted for 98-99% of basal tissue specific expression, and a proximal element (-422 to -36bp) accounted for 1-2% of activity. Both *cis* elements acted as enhancers, that is they conferred cell-type specific expression to a "neutral" viral promoter sequence independently of their position or orientation.

Although the studies of Nelson et al (1986) suggested that the distal enhancer region was relatively more important, the proximal enhancer element alone is sufficient to direct tissue-specific expression in its own

right. The functional importance of the proximal region of the PRL 5'-flanking DNA has been indicated by *in vitro* transcription experiments, using nuclear protein extracts to reconstitute transcription from a gene promoter element in a cell-free system. *In vitro* transcription experiments with GH<sub>3</sub> cell extracts suggested that the proximal regulatory elements were sufficient for tissue-specific expression of the rPRL gene: accurate initiation of transcription could be found using 420bp of 5'-flanking rPRL DNA, and whereas nuclear proteins from various cell types could direct transcription from viral gene promoters, only pituitary extracts could do so using the PRL promoter template DNA (Cao et al, 1987; Cao et al, 1988; Gutierrez-Hartman et al, 1987). Similarly, transient transfection studies have demonstrated that the proximal 250bp of the bovine PRL 5'-flanking sequence are adequate to confer tissue specific reporter gene expression (Camper et al, 1985). In summary, therefore, both the proximal and the distal enhancer elements are involved in tissue specific expression of the rPRL gene, though optimal enhancer activity probably requires synergism between the two.

#### 1.4.1.2 Cis/trans interactions

The first study of protein-DNA interactions in the rPRL gene suggested that pituitary-derived nuclear proteins were able to bind to two long stretches of 5'-flanking DNA extending as far as -4800bp upstream of the cap site (White et al, 1985). Subsequent studies have employed nuclease protection assays (protein binding protects DNA from nuclease digestion) to locate much more precisely the nucleotide sequences which bind trans-acting tissue-specific proteins.

Proximal protein binding sites Different studies and different techniques have yielded slightly differing results, but up to four protein binding

sites have been identified within the first 210bp 5' to the cap site, as shown in Figure 1.4 (Elsholtz et al, 1986; Schuster et al, 1988; Cao et al, 1987; Gutierrez-Hartman et al, 1987; Cao et al, 1988; Nelson et al, 1988; Lufkin et al, 1989). At least three of these proximal sites are pituitary specific as the protein binding is seen only with pituitary cell proteins and not with proteins from other tissues.

Of the four sites, the most proximal, between positions -30 and -78 has also been found to behave as an enhancer element which confers the transcriptional response to epidermal growth factor (EGF) and phorbol ester (Elsholtz et al, 1986). Using gel retardation assays it has been found that the electrophoretic mobility of this DNA element is reduced by protein binding, specifically with extracts from pituitary cells, but not from other cell types (Elsholtz et al, 1986; Schuster et al, 1988), again providing independent evidence for tissue-specific nuclear protein interactions with this region. In addition, an 18bp imperfect palindrome within this region appears to contain a TATA element which can initiate non-pituitary transcription from a site at -27bp from the usual initiation site. This may be prevented in pituitary cells by the presence of Pit-1/GHF-1 binding nearby, thus offering a further potential mechanism for cell-type specific gene expression (Barron et al, 1989).

The second protein binding site (-115/-130) may not be tissue-specific (Gutierrez-Hartmann et al, 1987) in that it may be found with non-pituitary protein extracts. However the fourth of these four proximal sites corresponds closely to a sequence identified by Lufkin & Bancroft (1987) which is able to direct gene transcription in hybrid pituitary-fibroblast fusion cells, but not in fibroblast cells alone. These data together provide direct evidence for the presence in pituitary cells of a diffusible

protein factor that can increase transcriptional rate, and whose effect is mediated by binding to 3 or 4 sites within the proximal enhancer region.

Distal binding sites Using DNase I footprinting, Nelson et al (1988) found that in addition to the four proximal protein binding sites described above, there were four distal sites between positions -1579 and -1718 in the rPRL gene (Figure 1.4). None of the total of eight sites were found with non-pituitary protein preparations in these studies, but interestingly the sites mutually competed for protein binding, suggesting that all eight bound a single protein factor; in addition protein binding was effectively reduced by competition with the tissue-specific enhancer sequences in the rat GH gene. One of these distal protein binding sites was independently demonstrated by Kim et al (1988), immediately adjacent to the oestrogen response element. The data of Nelson et al (1988) suggested the existence of a single pituitary-specific factor, named "Pit-1", which binds to enhancer regions of both PRL and GH genes. In fact it is now clear that Pit-1 is the same as the GH-specific factor GHF-I described by Lefevre et al, (1987); this gene has now been cloned independently by the two groups (Bodner et al, 1988; Ingraham et al, 1988), and is discussed in more detail below.

#### 1.4.1.3 Human prolactin gene

The tissue-specific regulation of the human prolactin gene has only been much more recently studied, but the organization of at least the proximal element of the 5' flanking region appears similar. Transfection studies have shown that 740bp of hPRL promoter DNA can direct tissue-specific expression of the CAT reporter gene in pituitary cells, but not in HeLa cells. DNase I footprinting studies have indicated pituitary-specific *trans* factor binding to three sites, corresponding to the highly homologous

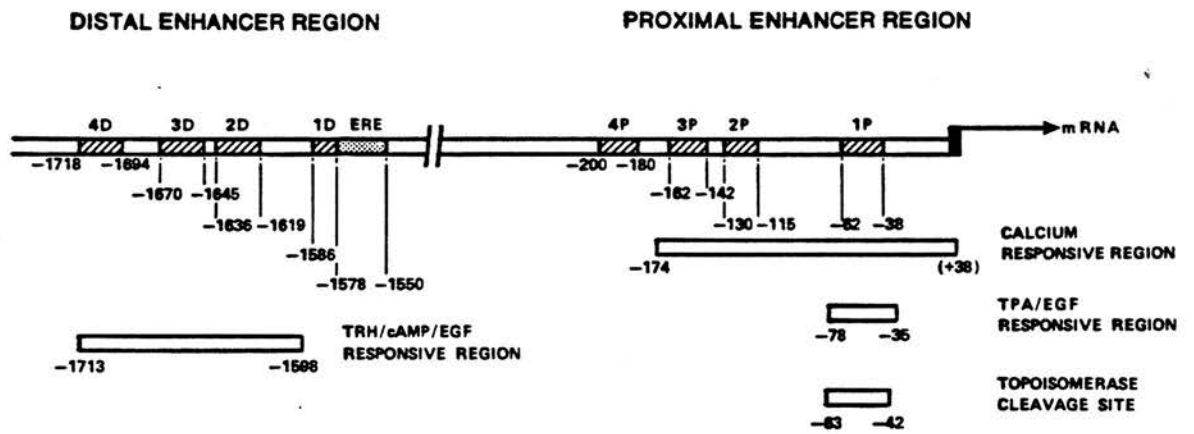


Figure 1.4

Diagram of proposed binding sites in the rat PRL gene for the pituitary-specific factor (Pit-1/GHF-1), shown as cross-hatched boxes 1P-4P and 1D-4D. The oestrogen response element (ERE) is shown as a stippled box, and putative response elements are indicated below.

sites 1,3 and 4 in the rat gene, except that "site 4" is slightly further upstream (Lemaigre et al, 1989). However it is worth noting that these studies employed nuclear proteins derived from rat pituitary cells, and not human tissue, and thus the evidence for a human Pit-1/GHF-1 protein is so far indirect. This question will be addressed in the gel retardation studies described in Chapter 7.

#### 1.4.2 GROWTH HORMONE

##### 1.4.2.1 Cis elements

Transfection studies using rat GH promoter-CAT fusion genes have shown that as little as 235bp of rat GH 5'-flanking sequence are able to confer cell-type specificity onto a reporter gene (Nelson et al, 1986). The degree of expression fell dramatically with deletion of more than 181bp 5' to the cap site, implying the presence of a tissue-specific enhancer element between -181 and -235bp; however neither elements extending from -235 to -146, nor from -181 to the cap site, were fully effective, suggesting that the putative enhancer element was relatively extensive. Independent transfection studies using much longer stretches of rat GH promoter and 5'-flanking sequences (1800bp) confirmed that CAT expression was restricted to pituitary cells, but this restriction of specificity was lost when 5' sequences were deleted down to the -309 or -183 positions (Larsen et al, 1986a). This suggested the presence of upstream repressor elements which normally prevented gene activation in non-pituitary cell types. Although these data conflict with those of Nelson et al (1986), in suggesting that tissue-specificity is not conferred solely by 235bp of 5'-flanking sequences, further data from Larsen et al (1986b) showed that sequences between -183 and -202 were able to confer thyroid hormone



responsiveness, but only in pituitary-derived cell lines. Thus there is evidence for overlap between these two aspects of gene regulation: a thyroid hormone response element (discussed in more detail below) may respond in a tissue-specific manner; or conversely, hormone responses may be mediated by interaction with tissue-specific *trans* factor binding, and a larger enhancer region consisting of a thyroid hormone response element may be functionally linked with tissue-specific elements (Ye et al, 1988).

#### 1.4.2.2 Cis/trans interactions

Gel retardation analysis has been used to demonstrate tissue-specific protein interactions with rat GH 5'-flanking sequences by Ye & Samuels (1987). Nuclear protein extracts from pituitary GC or GH4C1 cells selectively retarded the electrophoretic migration of -104/+7 and -236/-146 restriction fragments, whereas nuclear proteins from rat hepatoma or fibroblastic cells failed to do so.

More precise localization of protein-binding sites within rat GH 5'-flanking DNA has been undertaken with DNase I footprinting studies. As for the studies on the rPRL gene, the exact details vary slightly between different reports, but two consensus footprints may be deduced between positions -98 and -62 ("GC1"), and -140 and -106 ("GC2"), as summarised in Figure 1.5 (Ye et al, 1988; West et al, 1987; Catanzaro et al, 1987; Glass et al, 1987; de Groot et al, 1988). Other footprints have been shown further upstream (Glass et al, 1987; de Groot et al, 1988); a site at -220/-241, termed "GC3" has recently been shown to be pituitary-specific, but binds a different protein factor from the GC1 and GC2 sites (Guérin & Moore, 1988). The spatial relationship between the two proximal elements is important, and Ye et al (1988) showed that proteins must bind on the same side of the DNA helix to preserve full efficacy of this region,

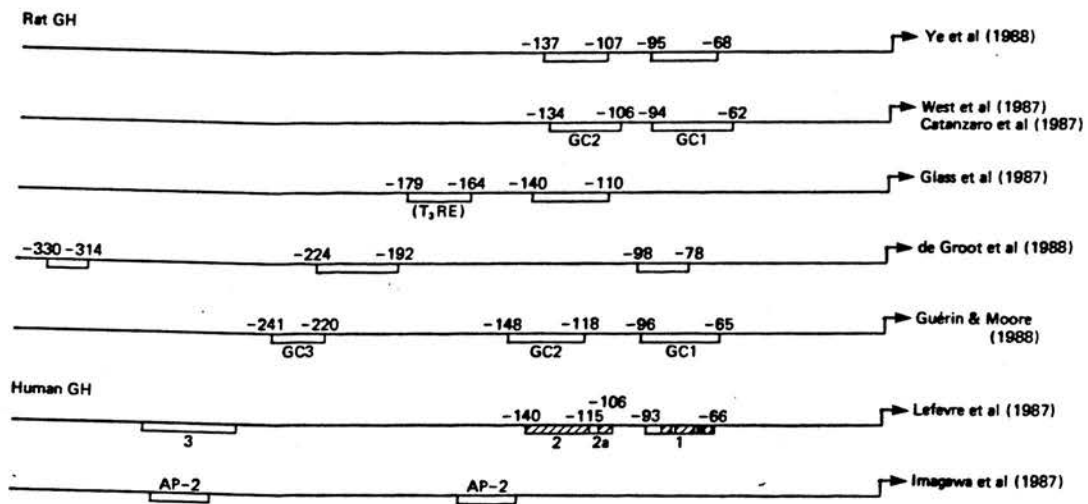


Figure 1.5

Diagram of proposed protein binding sites in the rat and human GH genes, as described by different authors.

suggesting that contact between two protein-DNA complexes is necessary.

#### 1.4.2.3 Human GH gene

Transfection studies of the human GH gene showed that 500bp of 5'-flanking DNA allowed expression of a reporter gene in rat GC or GH3 cells, but not in the non-pituitary Rat-1, HeLa or placental JEG-4 cell lines (Lefevre et al, 1987), suggesting the presence of a cell-type specific enhancer element within this region. Likewise Cattini et al (1986a), using deletion analysis, found that an element between -230 and -180 was necessary for pituitary-specific expression.

Using DNase I footprinting, Lefevre et al (1987) found three hGH sites binding proteins from rat GC cell extracts at -66/-93, -106/-140 and -254/-290; of these three sites, part of site 2 and all of the distal site 3 were also protected by HeLa cell extracts. Thus two pituitary-specific protein binding sites were identified (sites 1 and 2a; Figure 1.5), and they mutually competed for binding of a single protein factor which was named "GHF-1". Mutations within either site abolished GHF-1 binding. In fact both GHF-1 sites contain regions very highly homologous to the GC1 and GC2 sites described for the rGH gene promoter (West et al, 1987; Catanzaro et al, 1987) and have similar locations. GHF-1 is not the only protein involved however, and Imagawa et al (1987) have demonstrated footprints from binding of purified "Activator Protein-2" (AP-2) to two further sites in the hGH gene 5'-flanking region: AP-2 is a *trans* acting factor which is involved in intracellular messenger signalling, and is not a tissue-specific factor, and indeed the more distal site in fact coincides with the non-tissue-specific site 3 found by Lefevre et al (1987), as shown in Figure 1.5.

The functional importance of these protein-binding regions in maintaining

hGH gene transcription has been demonstrated by *in vitro* transcription experiments. Bodner & Karin (1987) found that 289bp of hGH 5'-flanking DNA was able to efficiently support transcription with pituitary GC cell nuclear protein extracts. HeLa cell extracts alone were much less efficient in supporting transcription from the hGH promoter template, but complementation with pituitary extracts increased transcriptional activity. Mutations in each of the two GHF-1 binding sites caused a severe reduction in transcriptional activity of the template.

#### 1.4.3 SUMMARY: FUNCTION AND NATURE OF PIT-1/GHF-1

For both the PRL and GH genes, in both man and rat, there is evidence for the existence of DNA sequences which are responsible for tissue-specificity of gene expression. Binding of nuclear proteins to these regions has been demonstrated, and appears to be responsible for establishing basal levels of gene transcription. Two sites within the rat and human GH promoter DNA are homologous and appear to bind the same protein *trans* acting factor (GHF-1, or GC1/GC2); eight sites upstream of the rPRL gene, and at least three sites for the hPRL gene also appear to bind a single *trans* factor, (Pit-1) and sequences of the GH gene compete for protein binding. Thus a single protein in both rat and human (Pit-1/GHF-1) may be responsible for directing expression of both genes and may also bind to the promoter of the TSH- $\beta$  gene (Nelson et al, 1988).

The cDNA encoding Pit-1/GHF-1 has recently been cloned (Bodner et al, 1988; Ingraham et al, 1988) and the sequence identity confirmed. The factor is now known to be a 33kD protein, which contains an amino-terminal 60-amino-acid region which is related to the "homeodomain" which characterizes a class of genes important in embryonic development in

*Drosophila*. It has therefore been described as a member of the "POU" family of homeodomain transcription factors (Herr et al, 1988; Mitchell & Tjian, 1989). At the carboxy-terminal a 76-amino-acid region is homologous to the mammalian transcription factor Oct-2, which regulates lymphoid-specific expression of immunoglobulin genes. Another member of the POU family, Oct-1, has a similar DNA recognition sequence, but has a ubiquitous tissue distribution. Minor changes in DNA recognition sequence are sufficient to change the tissue specificity of these transcription factors between pituitary and lymphoid cell types (Elsholtz et al, 1990). Detailed studies on the functional domains of Pit-1/GHF-1 (Theill et al, 1989) have shown that the homeodomain is sufficient for DNA-binding to the hGH gene promoter, and its activity is stimulated by the POU-specific domain. Transcriptional activity appears to be dependent on a separate domain, rich in hydroxylated amino-acids and named the STA domain (serine, threonine-rich activation domain). This feature is shared by Oct-2 and another member of the POU-domain family, *unc-86*.

There is disagreement on the crucial point as to whether Pit-1/GHF-1 protein can bind to both the PRL and GH promoters (Mangalam et al, 1989) or just the GH promoter (Castrillo et al, 1989; Theill et al, 1989; Karin et al, 1990). However, Pit-1/GHF-1 mRNA is expressed in lactotrophs and thyrotrophs as well as somatotrophs (Crenshaw et al, 1989). Affinity purified Pit-1/GHF-1 is able to direct transcription *in vitro* from both the PRL and GH promoters, and permanent transfection studies have shown that even low-level expression (as well as the very high level expression obtained with transient transfection) of the Pit-1/GHF-1 gene in heterologous HeLa cells will activate transcription of constructs containing either PRL or GH promoters (Mangalam et al, 1989; and see also

Ingraham et al, 1988). The most direct demonstration that *cis*-active elements binding Pit-1/GHF-1 are indeed important *in vivo* in directing tissue-specific expression has recently come from experiments using transgenic mice. Lira et al (1988) showed that upstream sequences of the rat GH gene were able to direct expression of a linked human GH structural gene predominantly in pituitary somatotrophic cells, but also in lactotrophs and thyrotrophs, while not in gonadotrophs or corticotrophs.

The fact that Pit-1/GHF-1 is found in three pituitary cell types suggests that some restrictive mechanism must exist to specify PRL or GH gene expression in their respective cell phenotypes. Transgenic mouse experiments have shown that the PRL upstream enhancers directed pituitary-specific reporter gene expression: either the proximal or the distal enhancer elements directed low-level expression, but when both elements were combined they interacted synergistically to direct high-level expression which was confined exclusively to lactotrophs. When the distal enhancer element was isolated from its flanking DNA, it directed reporter gene expression to thyrotrophs as well as lactotrophs (Crenshaw et al, 1989). A speculative model was therefore proposed in which the proximal and distal enhancer elements themselves could activate PRL gene expression in lactotrophs but restrict it in somatotrophs, while DNA elements flanking the distal enhancer region restricted PRL expression in thyrotrophic cells. However there may be other mechanisms in operation as well, determining the level of Pit-1/GHF-1 expression and activity: GH gene expression is extinguished when pituitary cells are fused with heterologous somatic L-cells, and this process is accompanied by loss of GHF-1 mRNA accumulation and protein production (McCormick et al, 1988). This suggests that a repressor factor restricts the tissue-specific expression of Pit-1/GHF-1

itself, and it is likely that more interacting factors will be discovered.

In summary, a current working hypothesis suggests that a single *trans*-acting factor directs cell-type-specific expression of three genes (PRL, GH and TSH- $\beta$ ) to three cell types within the pituitary, and that additional undefined DNA-protein interactions restrict the ultimate expression pattern to just one of these hormone genes in each case. The pattern of gene expression within a single cell type presumably reflects the integration of a variety of other signals such as thyroid hormone, glucocorticoids, sex steroids and peptide hormones. The proximity of Pit-1/GHF-1 sites to hormonally responsive regions in both genes is noteworthy, and it may be that some hormonal signals exert their effect by modifying the binding of tissue specific factors, or by covalent modification of *trans* factors that are already bound to DNA sequences, increasing or decreasing their ability to interact with the RNA polymerase II transcription initiation complex.

Immediately before submitting this thesis, two further important studies have been published on the nature of Pit-1/GHF-1, which both favour its role as a GH-specific transcription factor. Dollé et al (1990) have found that Pit-1/GHF-1 mRNA expression in the fetal mouse pituitary is confined to somatotrophs, apart from a transient period of lower level expression in lactotrophs, which then disappears. The expression in somatotrophs preceded that of GH itself by three days. Secondly, McCormick et al (1990) have analysed the Pit-1/GHF-1 promoter and found that it contains two CREB binding sites, but also a binding site for Pit-1/GHF-1 itself, and a site for a "somatotroph determining factor". Thus Pit-1/GHF-1 expression was cAMP-responsive and perhaps mediated cAMP regulation of GH production; once produced, it can maintain its own production, committing the cell's somatotrophic differentiation.



## 1.5 HORMONAL AND INTRACELLULAR REGULATION

Whereas the tissue-specific expression of the PRL and GH genes involve a common mechanism, at least in part, their hormonal regulation differs markedly. A large number of hormonal signals may act upon given cell types, and in principle these might act either independently or by modulating tissue-specific control mechanisms. Thus, hormonally induced *trans*-acting factor activation may either have a direct effect on "dedicated" hormone-responsive DNA sequences, or an indirect effect by modulating tissue-specific protein (for example Pit-1/GHF-1) interactions with DNA. The latter possibility has recently been proposed to explain the action of the glucocorticoid receptor on the PRL gene (Adler et al, 1988).

Oestrogen and thyroid hormones exert their effects by binding to specific nuclear receptors which have been shown to be DNA-binding proteins, and much of our present understanding of receptor-DNA interactions derives from exciting recent work on nuclear receptor structure (reviewed by Evans, 1988). But peptide hormones and dopamine, which bind to membrane receptors, must act through intermediate intracellular signalling systems via "second messengers" (Berridge, 1985; Brown et al, 1985), and the ways in which these act to regulate gene transcription is much less clear. For these substances, the relevant pathways linking second messenger formation to *trans*-activating proteins have yet to be characterized: do the various intracellular signals linked to given membrane receptors initiate a cascade which converges onto a final common pathway in the nucleus, or is the variety of second messenger signals an important channelling mechanism allowing integrated effects on a gene of a wide variety of different transactivation proteins?



### 1.5.1 PROLACTIN

#### 1.5.1.1 Nuclear hormone receptors

PRL gene transcription is affected by a wide variety of hormones (Table 1.1); for some of these, hormone responsive elements have been clearly identified in the 5'-flanking region of the PRL gene.

Oestrogen 17- $\beta$ -oestradiol causes increased accumulation of PRL mRNA in rat pituitary cells *in vitro* (Stone et al, 1977) and *in vivo* (Tong et al, 1989), and this effect is due to a rapid stimulation of the rate of gene transcription (Maurer, 1982a; Shull & Gorski, 1984; Waterman et al, 1988). There is little or no effect with progesterone or androgens, although androgens may reduce the oestrogen-induced stimulation *in vivo* (Tong et al, 1989). The stimulatory effect of oestrogen is exerted by direct binding of oestrogen receptor (ER) to the rPRL gene (Green & Chambon, 1986; Evans, 1988), and an oestrogen response element (ERE) in rat PRL 5'-flanking DNA has been located more than 1500bp distant from the transcription initiation site (Maurer, 1985; Maurer & Notides, 1987; Waterman et al, 1988; Somasekhar & Gorski, 1988). This ERE is close to a region of DNase I hypersensitivity, which may be important in the mechanism of transcriptional activation (Durrin et al, 1984; Durrin & Gorski, 1984); but the ERE is also immediately adjacent to one of the four distal Pit-1/GHF-1 binding sites (Kim et al, 1988; Nelson et al, 1988) suggesting possible interactions between oestrogen receptor and the Pit-1/GHF-1 protein.

Glucocorticoids Dexamethasone inhibits PRL gene transcription by an action exerted within the proximal 5'-flanking region of the rat and bovine gene promoters (Camper et al, 1985; Adler et al, 1988; Sakai et al, 1988; Somasekhar & Gorski, 1988). The bPRL gene promoter contains multiple footprinting sites for purified glucocorticoid receptor, but these sites

	<u>PRL</u>	<u>rGH</u>	<u>hGH</u>
<u>Hormones</u>			
Oestrogen	+		
Thyroid hormone	-	+	-
Glucocorticoid	-	+	+
Vitamin D	+		
TRH	+	-	
GRF		+	+
Dopamine	-		
Somatostatin		-	
VIP	+		
GABA	-		
Insulin	+	+/-	
IGF-I		-	-
EGF	+		
bFGF	+		
<u>Intracellular signals</u>			
Cyclic AMP	+	+	+
Calcium	+	+	
Phorbol ester	+		

Table 1.1

Summary of known effects of hormones and intracellular messenger substances on gene transcription or mRNA accumulation. + indicates stimulation, and - indicates inhibition.

appear to differ from positive GRE consensus sequences (Sakai et al, 1988). However interestingly it was found that an inhibitory effect of oestrogen on the proximal region of the rPRL promoter could be expressed in specially manipulated circumstances (by inducing over-expression of the ER); it was then shown that the inhibitory effect of the glucocorticoid receptor (GR) and the overexpressed ER was not exerted by their DNA-binding domain (Adler et al, 1988). Thus, the stimulatory and inhibitory effects of these receptors were exerted by different parts of the molecule, and the negative effects did not involve direct protein-DNA interaction but most probably indirect effects on other DNA-binding proteins.

Various protein-protein interactions have recently been proposed for glucocorticoid receptor action on other genes, where the spatial arrangement of glucocorticoid response elements is critical and are frequently clustered with other *trans* factor binding sites (Schüle et al, 1988). Indeed in the pituitary glycoprotein  $\alpha$ -subunit gene, Akerblom et al (1988) found overlap between the cAMP responsive region of the gene promoter and the GR binding element, and suggested that negative regulation could result from interference of a hormone-receptor-DNA complex with other positive *trans*-activating proteins.

Thyroid hormone and Vitamin D Two other nuclear receptors, binding  $T_3$  and vitamin D respectively, are closely related (Evans, 1988), and seem likely to exert effects on the PRL gene, but in contrast to the rapid advances in knowledge of GH gene regulation by  $T_3$ , less is known about their mechanism of action on the PRL gene. It has been shown that  $T_3$  inhibits PRL mRNA accumulation in rat pituitary cells (Maurer, 1982b; Stanley & Samuels, 1984), and that the weak thyroid hormone agonist drug phenytoin has similar effects (Davis et al, 1986). Transient transfection of GH cells with

plasmid expression vectors containing the c-erb-A  $\alpha$  and  $\beta$  genes (encoding  $T_3$  receptors) have shown  $T_3$ -inducible inhibition of expression of a co-transfected PRL promoter-reporter gene construct, providing the first evidence that c-erb-A proto-oncogenes can directly affect the PRL (and also GH) gene expression (Forman et al, 1988).

However the overall picture is complex. Transient transfection assays in  $GH_3$  cells using deletants of the rPRL promoter have disclosed both positively and negatively acting  $T_3$ -response elements in the distal (-1530/-1565bp) and proximal (-292/-38bp) enhancer regions respectively (Day & Maurer, 1989a). Furthermore, different GH cell lines display different responses of transfected rPRL-CAT constructs to  $T_3$ , with stimulation in  $GH_4C1$  cells, but inhibition in  $GH_1$  cells (Stanley, 1989). Thus the rPRL gene possesses countervailing response elements, and also that different cells presumably possess different *trans*-acting factors, the balance of which determine the ultimate cellular response to  $T_3$ .

Vitamin D has been shown to stimulate cytoplasmic accumulation of rPRL mRNA (Wark & Tashjian, 1983) and this stimulation is antagonized by glucocorticoid (Wark & Gurtler, 1986); however little more is so far known about the interaction of Vitamin D receptor with this target gene. It seems reasonable to speculate that nuclear receptors for  $T_3$  and vitamin D may interact with DNA or other *trans* factors in a manner similar to the ER and GR, and indeed it has been found that  $T_3$ -receptor may bind to oestrogen response elements in a transcriptionally inactive form, to decrease the overall level of gene transcription (Glass et al, 1988).

#### 1.5.1.2 Peptide and dopamine regulation

Thyrotrophin-releasing hormone (TRH) stimulates rPRL gene transcription within minutes, and increases cytoplasmic mRNA accumulation (Laverriere et

al, 1983; Murdoch et al, 1983; White & Bancroft, 1983). In addition TRH increases the half-life of cytoplasmic mRNA from 17 to 27 hours (Laverriere et al, 1983). Similar stimulation is seen with epidermal growth factor (EGF; White & Bancroft, 1983; Murdoch et al, 1985b). Supowit et al (1984) showed by transfection studies that 3000bp of 5'-flanking DNA conferred EGF responsiveness onto a reporter gene, and for the bovine PRL gene the proximal 250bp were sufficient to allow stimulation by TRH and EGF (Camper et al, 1985), implying that the proximal enhancer region contains the necessary sequences for recognition of the intracellular signals generated by these two peptide hormones. Vasoactive intestinal peptide (VIP) also elevates PRL mRNA levels in pituitary cells (Carrillo et al, 1985). Dopamine rapidly inhibits PRL gene transcription, as does the dopaminergic drug bromocriptine (Maurer, 1980, 1981, 1982c; and see Chapter 2), consistent with the well known dopaminergic suppression of prolactin secretion. Basic fibroblast growth factor markedly stimulates rPRL mRNA accumulation but has no effect on GH mRNA (Black et al, 1989), and finally,  $\gamma$ -amino-butyric acid (GABA) inhibits and insulin stimulates PRL mRNA accumulation (Loeffler et al, 1985; Prager et al, 1988).

These various hormones are thought to act through different intracellular mechanisms: TRH stimulates the hydrolysis of membrane phospholipids resulting in protein kinase C activation and calcium mobilization, and leading to phosphorylation of cytoplasmic and nuclear proteins (Drust & Martin, 1982; Murdoch et al, 1982; Gershengorn, 1986;); EGF activates a transmembrane protein kinase (Stoschek & King, 1986); VIP activates adenylate cyclase (Gourdjil et al, 1979), which results in phosphorylation of a different group of proteins than with TRH action (Drust et al, 1982; Murdoch et al, 1983). Thus the rPRL gene is likely to be controlled by a

number of interacting intracellular messenger systems (Bancroft et al, 1985; Murdoch et al, 1985a).

#### 1.5.1.3 Intracellular messenger pathways

Cyclic AMP Cyclic AMP has been shown to increase PRL gene transcription (Maurer, 1981), and forskolin (which activates adenylate cyclase) increases cytoplasmic mRNA accumulation (Murdoch et al, 1982b; Dave et al, 1987).

Cyclic AMP responsive elements (CREs) have been defined for various genes including the pituitary glycoprotein  $\alpha$ -subunit (Delegeane et al, 1987), VIP (Tsukada et al, 1987) and somatostatin (Montminy & Bilezikjian, 1987). The best understood system involves the *trans*-acting factor CREB (CRE binding protein) a 43kD protein which binds to an octameric CRE (Montminy & Bilezikjian, 1987; Hoeffler & Habener, 1990). The DNA binding and transcriptional efficacy of this protein may be regulated by its phosphorylation by cAMP-dependent protein kinase (Yamamoto et al, 1988). While cAMP has clear effects on rPRL gene expression, these probably do not involve CREB, as there are no classical CREs in the relevant enhancer regions. However a second well documented system of cAMP regulation is mediated by the *trans*-acting factor AP-2, which has two binding sites in the hGH promoter (Imagawa et al, 1987) and a potential site in the rPRL promoter. The cAMP induction was seen with promoter sequences as short as 127bp, which had little basal promoter activity (Keech & Gutierrez-Hartmann, 1989). Interestingly, however, another study showed that basal as well as cAMP induced PRL gene expression involves both isozymes of the catalytic subunit of protein kinase A (Maurer, 1989), and co-expression of the protein kinase inhibitor gene in transfected cells reduces PRL promoter induction by EGF, phorbol ester and oestradiol (Day et al (1989). Thus

there is evidence that cAMP may interact with the mechanisms for tissue-specific basal gene expression.

Calcium The intracellular signals known to mediate TRH action on PRL secretion are intracellular calcium and protein kinase C. Intracellular calcium appears to be crucial for PRL gene expression: GH<sub>3</sub> cells grown in low-calcium medium respond to addition of calcium by a dramatic rise in both PRL synthesis and mRNA accumulation (White et al, 1981), which occurs within 10 hours. On the other hand, removal of extracellular calcium by EGTA rapidly reduces PRL mRNA levels to undetectable levels, whereas GH mRNA levels show a smaller, slower fall (Glick & Bancroft, 1985). Calcium influx into cells induced by the dihydropyridine calcium channel agonist Bay K 8644 can stimulate PRL gene transcription rate (Hinkle et al, 1988; Laverriere et al, 1988), again suggesting an effect of intracellular calcium concentrations *per se*. However, studies utilising calcium ionophores have been difficult to interpret, with conflicting results: Murdoch et al (1985b) found no effect of either ionomycin or A23187 alone on PRL gene transcription rate although they did synergise with the effect of the phorbol ester TPA; in contrast Laverriere et al (1988) found that both these agents increased transcription. A recent detailed study of these agents has suggested that these ionophores may have non-specific effects on gene transcription due to disruption of normal calcium gradients between different intracellular compartments (White et al, 1989), rather than the more specific responses seen using the dihydropyridine agents that affect voltage sensitive channels only.

A requirement for calcium has further been demonstrated by studies using calcium channel blocking agents such as verapamil, nifedipine or diltiazem, and calcium antagonists such as cobalt (Murdoch et al, 1985b; Davis et al,



1988a; Hinkle et al, 1988). Studies with calmodulin-antagonist drugs have suggested that PRL mRNA accumulation is calmodulin-dependent (Murdoch et al, 1985b; White, 1985; Bancroft et al, 1985). The possible molecular mechanisms for calcium/calmodulin action in the induction of PRL gene transcription are discussed in detail in Chapter 5. However one particular mechanism may involve the calcium/calmodulin-dependent nuclear protein, DNA topoisomerase, which has DNA-unknotting and relaxing activity in vitro (White & Preston, 1988). DNA topoisomerase interacted with three sites within 3000 bases of PRL 5'-flanking DNA, and one of these sites was within the first 100bp (White & Preston, 1988). Further recent evidence from transfection studies have suggested that a "calcium regulatory element" may lie within the first 174 bases of the 5' flanking DNA (Jackson & Bancroft, 1988).

Protein kinase C PRL gene transcription is stimulated by activation of protein kinase C with tumour-promoting phorbol esters such as 12-O-tetradecanoyl phorbol 13 acetate (TPA; Murdoch et al, 1985b). Studies of rPRL gene 5'-flanking DNA have demonstrated an upstream TPA response element (Supowit et al, 1984). This in fact coincides exactly with the EGF-response element, and lies between -78 and -35 bases upstream from the transcription start site (Elsholtz et al, 1986). This region of DNA is clearly of major interest as it forms part of a putative calcium-regulatory element and a site of DNA topoisomerase action (Jackson & Bancroft, 1988; White & Preston, 1988), but also represents the first proximal binding site for Pit-1/GHF-1 (Nelson et al, 1988).

Great progress has recently been made regarding protein kinase C effects on the transcription of other genes, notably the glycoprotein  $\alpha$  subunit, which are mediated by the *trans*-acting factor AP-1, encoded by the *jun*



proto-oncogene (Lee et al, 1987; Curran & Franza, 1988; Hoeffler et al, 1989). However it remains unclear whether AP-1 is implicated in phorbol ester stimulation of PRL gene expression, or whether other mechanisms are involved.

In summary, responses to various intracellular messenger systems seem to involve proximal 5'-flanking DNA sequences in the rPRL gene, and the relevant response elements may coincide with binding sites for Pit-1/GHF-1. However such response elements may be multiple, and Day & Maurer (1989b) have recently shown that the distal enhancer region (-1713/-1495) conferred regulation by TRH, EGF and cAMP (though interestingly, not phorbol ester) onto a reporter gene. These responses were equal to those seen with the proximal enhancer, and a synergistic interaction was found between these agents and oestradiol, suggesting co-operation between DNA-bound *trans*-acting factors.

#### 1.5.2 GROWTH HORMONE

The number of probable hormonal regulators of the GH gene is rather smaller than for PRL (Table 1.1). The best studied of these regulators is thyroid hormone (reviewed by Samuels et al, 1988), which is well known to be a major regulator of GH secretion in vivo, at least in the rat; the peptide hormones, growth hormone releasing factor (GRF), somatostatin and IGF-I have so far received much less attention.

##### 1.5.2.1 Thyroid hormone

Rat GH gene transcription is induced by  $T_3$  by up to ten-fold in vivo and in vitro in the pituitary GC cell line (Spindler et al, 1982; Nyborg et al, 1984; Yaffe & Samuels, 1984). This induction coincides with  $T_3$  receptor occupancy and alterations in chromatin structure (Nyborg et al, 1986). The

c-erb-A proto-oncogene, which encodes a  $T_3$  nuclear receptor has now been shown in co-transfection experiments to induce GH gene expression (Forman et al, 1988).

The *cis* elements involved in this  $T_3$  regulation have been more precisely located by transfection studies using various lengths of rat GH gene promoter linked to reporter genes. In a series of experiments with cells that had stably integrated the exogenous constructs into their genome,  $T_3$  induction could be observed with progressively smaller promoter fragments from 1800bp (Casanova et al, 1985) down to 235bp of 5'-flanking DNA (Crew & Spindler, 1986). Further analysis showed the simultaneous presence of a positive and a negative thyroid response element (Wight et al, 1987); the first of these behaved as an enhancer (at position -254/-241) but the second did not, and overlapped with the TATA box (position -46/-21). Later studies with staged deletions of 5' and 3' regions in rGH fragments (from which the negative element had been deleted), mapped the borders of the positive element to position -194/-169 (Wight et al, 1988) corresponding closely to a  $T_3$  receptor footprint described by Koenig et al (1987), and which contains part of a site that binds the c-erb-A proto-oncogene translation product (Glass et al, 1987).

In transient expression studies of transfected cells (Larsen et al, 1986a; Flug et al, 1987), the data differed slightly from the results described above, perhaps because the constructs are not integrated in the host cell genome and have a higher copy number of constructs per cell, which might compete for binding of some regulatory factors. Larsen et al (1986a) found that sequences between positions -183 and -202 were able to confer thyroid hormone responsiveness, but only in pituitary cell types, indicating that a thyroid hormone response element itself may have tissue-

specific characteristics. Ye et al (1988) proposed that two interacting tissue-specific *cis* elements (at -137/107 and -95/-65) are functionally linked with the T<sub>3</sub> response element to act as an enhancer-like unit which confers both cell-type-specific and T<sub>3</sub>-regulated gene expression. Both were necessary to confer regulated expression to heterologous promoters, and it was suggested that the T<sub>3</sub>-receptor-DNA complex is able to enhance the function of the cell-specific elements to increase the level of rat GH gene expression. Finally, gel retardation and DNase I footprinting studies have shown that purified T<sub>3</sub>-receptor can bind to upstream regions of the GH gene, and that there may in fact be multiple sites which can interact with receptor within the region -530/+7 (Ye & Samuels, 1987; Apriletti et al, 1988; Lavin et al, 1988; DeGroot et al, 1988).

#### 1.5.2.2 Glucocorticoids

Transcription of the endogenous rat GH gene is induced by five to twenty-fold in the GH cell line (Martial et al, 1977; Evans et al, 1982; Wegnez et al, 1982). Initial attempts at studying the mechanism of glucocorticoid action were undertaken using heterologous (non-pituitary) recipient cells which had stably integrated the transfected rGH gene into their genome (Doehmer et al, 1982; Kushner et al, 1982; Karin et al, 1984; Miller et al, 1984). A rGH promoter region as short as 248bp was able to confer glucocorticoid induction to a reporter gene in heterologous cells (Heiser & Eckhardt, 1985), but in transient transfection analyses using rGH 5'-flanking DNA linked to a reporter gene in pituitary cells, Crew & Spindler (1986) and Flug et al (1987) found only minimal glucocorticoid effects. Birnbaum & Baxter (1986) found that a promoterless rGH gene was glucocorticoid inducible, with mRNA of a nearly correct size, suggesting that glucocorticoid response elements (GREs) were contained downstream, not

upstream, from the transcription initiation site. A GRE has been described in the first intron of the human GH gene (Slater et al, 1985), and although such a site has not yet been shown in the rat GH gene, its first intron does not contain typical GRE sequences.

#### 1.5.2.3 Peptide hormone regulation and intracellular messenger systems

Hypothalamic GRF stimulates rat GH gene transcription in primary cell cultures (Barinaga et al, 1983; Gick et al, 1984; Barinaga et al, 1985), and also in transgenic mice overexpressing the human GRF precursor gene (Hammer et al, 1985a). Somatostatin reduces rat GH mRNA accumulation (Wood et al, 1987), although another study showed no immediate effects on transcriptional rate (Barinaga et al, 1985). Insulin blocks  $T_3$  stimulation of rGH secretion (Melmed & Slanina, 1985) and may inhibit GH mRNA accumulation (Melmed et al, 1985a; Yamashita & Melmed, 1986), but depending on culture conditions, other workers have found that insulin may also exert stimulatory effects (Isaacs et al, 1987a). Ectopic hypersecretion of GH by transplanted tumours in the rat has been shown to inhibit GH mRNA accumulation in vivo (Yamashita et al, 1986a), and this autoregulation probably involves negative feedback from insulin-like growth factor 1 (IGF-1; Yamashita & Melmed, 1987).

Transcription of the rat GH gene may be modulated independently of GH release: GRF stimulation of GH release is highly dependent upon extracellular calcium, whereas both basal and GRF-stimulated GH gene transcription are much less so (Barinaga et al, 1985). Rat GH mRNA accumulation is somewhat inhibited by removal of extracellular calcium but this effect is much less marked than for PRL mRNA (Gick & Bancroft 1985; Bancroft et al, 1985). GRF is thought to exert its effect on somatotrophs through

stimulation of adenylate cyclase, and its effects on rat GH gene transcription may be mimicked by exogenous cAMP (Clayton et al, 1986) and adenylate cyclase activation, but not by activation of protein kinase C or cell depolarization, which stimulate only secretion of GH (Barinaga et al, 1985; Morita et al, 1987).

#### 1.5.2.4 Human GH gene

Analysis of stable transformants obtained by transfection of the human GH gene with 500bp of promoter sequences into GH<sub>3</sub> cells (Cattini et al, 1986b) has shown that T<sub>3</sub> inhibits hGH gene transcription while stimulating that of the rat GH gene. However this T<sub>3</sub> inhibition is not clearly linked to the hGH promoter as it could not be shown in transient expression studies using the CAT reporter gene (Cattini & Eberhardt, 1987; Brent et al, 1988). The endogenous gene may not always behave in a similar fashion to a transfected construct. Transcriptional control of the endogenous human GH gene has been the subject of limited study, using tissue obtained from surgically removed pituitary somatotroph adenomas. Isaacs et al (1987b) found stimulation of hGH gene mRNA accumulation by dexamethasone, but no effect of T<sub>3</sub>, in contrast to the inhibitory effects of T<sub>3</sub> seen in 7 of 11 rat GC cell lines stably transfected with a human GH gene construct. These differences between transfected and endogenous genes, and the variability of the transfection data, suggest that the cellular environment is critically important in determining the ultimate response to a hormonal stimulus.

Although a T<sub>3</sub> response element has not yet been precisely identified in the human gene, T<sub>3</sub> receptor from lymphoblastic IM-9 cell nuclear extracts is able to bind specifically between positions -129 and -290 in the 5'-flanking region of the gene (Barlow et al, 1986), a position similar to

that of the positive  $T_3$  response element in the rat gene. Glucocorticoids stimulate expression of the transfected human GH gene (Robins et al, 1982; Cattini et al, 1986b; Brent et al, 1988) similar to their effect on the rat gene, and glucocorticoid response element has been demonstrated within the first intron of the gene, that is, downstream of the transcription initiation site (Eliard et al, 1985; Moore et al, 1985; Slater et al, 1985). A weaker glucocorticoid receptor binding site found within the promoter region (Eliard et al, 1985) could be responsible for the hGH-CAT response to the hormone (Cattini & Eberhardt, 1987).

Little work has been reported on the effects of peptide hormones on the human GH gene, but Yamashita et al (1986b) showed suppression of hGH mRNA levels by IGF-1 using cultured cells from a human pituitary adenoma; IGF-1 also prevented GRF-induced stimulation of GH mRNA levels. The intracellular messenger systems which may regulate GH gene transcription in the human pituitary are not yet known, but Brent et al (1988) showed that the transfected hGH promoter was cAMP-responsive. The hGH promoter contains no CREB binding sites, but its two AP-2 sites are not required for cAMP induction of reporter gene expression, in transient transfection assays (Dana & Karin, 1989), and thus the cAMP induction may involve Pit-1/GHF-1, or perhaps another novel cAMP response element. This aspect of hGH gene regulation is explored in the studies described in Chapter 4 of this thesis.

#### 1.5.3 SUMMARY: INTRACELLULAR CONTROL OF TRANSCRIPTION

For both the PRL and GH genes, a wide variety of hormones and intracellular signalling systems appear to have significant transcriptional effects. Whereas basal tissue-specific expression of the PRL or GH genes

requires the interaction of Pit-1/GHF-1 with defined sites in their promoters, modulation of a given level of expression may involve additive effects of adjacent or distant hormone response elements, or modification of Pit-1/GHF-1 or other factors already bound to their cis elements.

For the DNA-binding nuclear hormone receptors, the molecular basis of their effects is increasingly well understood, with evidence for both direct protein-DNA effects, and also for indirect protein-protein-DNA interaction. The picture is much less clear for the effects of intracellular second messenger systems. However a number of interesting models have recently been proposed for cAMP action on other genes, which may be relevant (reviewed by Roesler et al, 1988). In a cell-free system the transcription of the urokinase plasminogen activator gene is enhanced by addition either of cAMP itself or of the purified kinase catalytic subunit (Nakagawa et al, 1988). Thus a model may be proposed whereby cAMP (formed as a result of membrane receptor activation) activates a kinase, resulting in dissociation of its catalytic subunit: this is then translocated to the nucleus and phosphorylates a positive nuclear trans-acting factor to enhance gene transcription by RNA polymerase II. The *trans*-acting factor CREB, whose transcriptional activity may be enhanced by cAMP-dependent phosphorylation (Yamamoto et al, 1988), fits into this model very neatly, although there may be other potential mechanisms in addition. Very recently, it has been found that CREB may activate transcription of Pit-1/GHF-1, whose increased level of expression would then enhance GH gene expression (McCormick et al, 1990), showing that even in this relatively simple scheme, more than one transcription factor may be involved.

It is now recognised that many proto-oncogene products are directly involved in intracellular signalling pathways and the regulation of gene



transcription (Berridge, 1986), and some such proteins are now implicated in the expression of the prolactin and GH genes, apart from the well known example of the  $T_3$  receptor, encoded by the proto-oncogene *c-erb-A* (Sap et al, 1986; Weinberger et al, 1986). It has been shown that GRF, acting via cAMP, can stimulate somatotroph cell division and also rapidly increases levels of *c-fos* mRNA (Billestrup et al, 1986; Billestrup et al, 1987). The function of the *fos* protein in the pituitary is not yet known, but there is now good evidence that it interacts, probably by dimerization, with the proto-oncogene product, *c-jun*, which encodes "activator protein 1" (AP-1), a recognised transcription factor which is responsive to activation of protein kinase C (Lee et al, 1987; Chiu et al, 1988; Curran & Franza, 1988; Hoeffler et al, 1989; Karin, 1989; Mitchell & Tjian, 1989). Another such factor, AP-2, appears to be linked both to protein kinase C and to cAMP (Imagawa et al, 1987), and it will be interesting to learn whether the potential AP-2 binding sites in the prolactin and GH gene promoters are involved in intracellular control, and indeed whether they may interact with Pit-1/GHF-1 binding sites.

## 1.6 CONCLUSIONS

In this background review, I have discussed the tissue-specific and intracellular mechanisms for the regulation of the PRL and GH genes. The cell-type-specification of expression probably involves more than one pituitary-specific nuclear protein, and the role of Pit-1/GHF-1 has been discussed. In terms of the mechanisms of hormonal control of PRL and GH expression, the actions of steroid, thyroid hormone and peptide hormones on gene transcription are rapidly being clarified. In particular, many of the relevant DNA response elements are now identified, and in some cases the



*trans*-acting factors are known. Interestingly, the response elements involved in hormonal control of these two genes are closely related to Pit-1/GHF-1 binding sites, if not coincidental, thus the mechanisms of intracellular regulation appear to impinge on those of tissue-specificity. A general speculative model may perhaps be proposed whereby the basal level of gene transcription is determined by constitutive tissue-specific factor binding, and the hormonal stimulation or inhibition of transcription is superimposed, mediated by interactions between Pit-1/GHF-1 and other hormone-regulated or 2nd messenger-regulated nuclear proteins such as CREB, AP-1 or others yet to be identified.

The potential importance of such mechanisms in the pathogenesis of pituitary disease is unknown, but abnormalities of hormone gene transcriptional control may be involved. An approach to this topic forms the subject of much of this thesis.

## CHAPTER 2

### DOPAMINERGIC REGULATION OF RAT PROLACTIN

#### mRNA ACCUMULATION

#### 2.1 INTRODUCTION

Prolactin (PRL) secretion and gene transcription are inhibited by dopamine and dopaminergic drugs such as bromocriptine, and the PRL-inhibitory effect of bromocriptine has been of dramatic benefit clinically in the treatment of PRL-secreting pituitary tumours. In addition to reducing serum PRL levels, both bromocriptine and the newer ergot derivative pergolide have been shown to reduce the size of large prolactinomas (McGregor et al, 1979; Kendall-Taylor et al, 1982; Franks & Jacobs, 1983). The reduction in size of these tumours may be dramatic (Clayton et al, 1985), and although the mechanisms for such shrinkage are not understood, a close relationship appears to exist between the control of hormone production and that of tumour growth.

Very little is known about the dopaminergic regulation of endogenous PRL gene transcription in human pituitary tissue, and this will form the subject of the studies described in Chapter 3. However before presenting this work, and in order to demonstrate the application of a simple hybridization assay to such studies on normal pituitary tissue, this Chapter will describe the effects of three different dopamine agonist agents on cultured rat pituitary cells.

The clinical use of bromocriptine and pergolide has become well-established, but the frequency of adverse effects has led to the recent development of two new agents, CV 205-502 and CQP 201-403 (Sandoz

Pharmaceuticals). CQP 201-403 is a propylergoline and CV 205-502 a benzo[g]quinoline (Nordmann & Petcher, 1985), and while both agents are ergot derivatives, their structure differs substantially from bromocriptine (Figure 2.1), and thus their effects and potency might be expected to differ. Both drugs in fact appear to be more potent than bromocriptine in lowering serum PRL *in vivo* in both normal subjects and in hyperprolactinaemic women (Grevel et al, 1986; Rasmussen et al, 1987; Hanssen et al, 1988; Rasmussen et al, 1988).

Both CV 205-502 and CQP 201-403 inhibit PRL release by normal rat pituitary tissue and also human prolactinoma tissue *in vitro*, as studied by the perfusion technique (Venetikonou et al, 1987). These studies showed that in comparison to the transient inhibitory effect of dopamine, both agents caused profound and prolonged inhibition of PRL secretion, while there was little or no effect on growth hormone (GH) release. Thus it is clear that these newer dopamine agonists reduce prolactin release, but there is so far no information as to possible effects on hormone synthesis, which may be important if the drugs are to be of value in the long term treatment of hyperprolactinaemia or in attempts to reduce the size of large prolactinomas.

The effects of both agents on PRL mRNA accumulation were compared with those of bromocriptine in rat pituitary tissue, using the cytoplasmic dot hybridization method developed by White & Bancroft (1982). This technique has been extensively used for the study of pituitary hormone gene expression *in vitro* (Carrillo et al, 1985; Clayton et al, 1986; Davis et al, 1986; Preston & White, 1988), and it is simple and relatively rapid, allowing comparison of mRNA levels in multiple tissue samples.

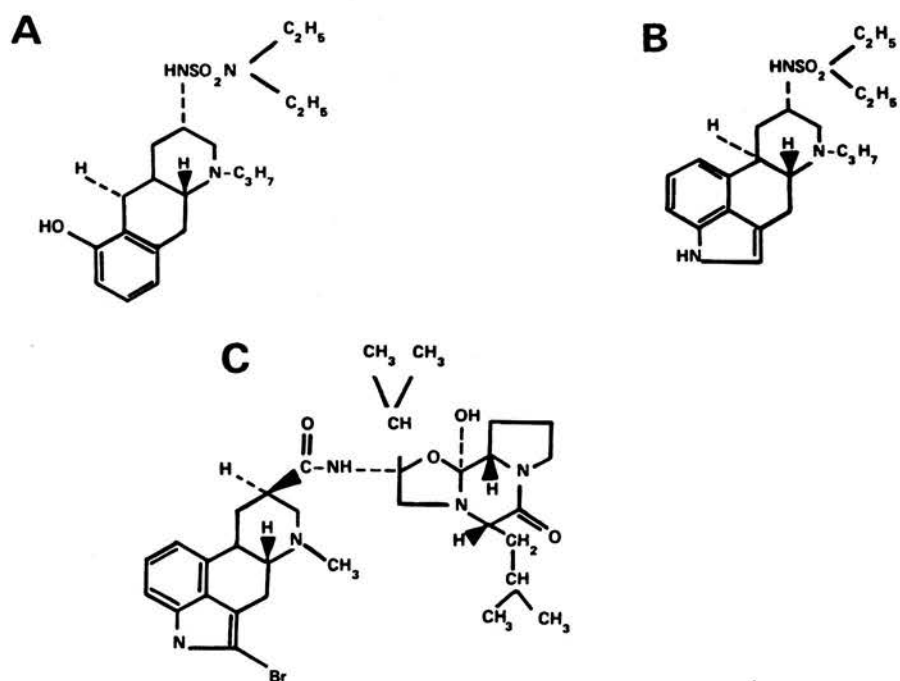


Figure 2.1 Structures of CV 205-502 (A), CQP 201-403 (B) and bromocriptine (C).

## 2.2 METHODS

For these experiments the use of the clonal GH cell lines was not possible as these cells lack functional dopamine receptors. Normal rat pituitary tissue was therefore required, and for these studies primary monolayer cell cultures were prepared from normal rat pituitary glands as described in Appendix A1.1. Briefly, up to 10 male rat pituitary glands were chopped into small fragments before enzymatic dispersion using collagenase and hyaluronidase. The cell suspension was washed free of enzyme solution, the cells counted, and plated onto 24-well cell culture plates at a density of approximately  $5 \times 10^5$  cells/well.

The cells were allowed to attach to the culture dishes for several days, and were then transferred to serum-free medium before treatment for 24-hour periods with either CV 205-502, CQP 201-403 or bromocriptine (Sigma Chemical Company, Poole, Dorset, UK). All three drugs were initially dissolved in ethanol, before dilution in culture medium. At the end of treatment periods, medium was removed for radioimmunoassay of released hormone (Appendix A4.1), and cells were scraped from culture wells for preparation of cytoplasmic samples.

Messenger RNA concentrations were estimated using the technique of cytoplasmic dot hybridization. The procedure is described in detail in Appendix A3.1. Briefly, cytoplasmic samples were prepared from the cultured cells, and applied in duplicate onto a hybridization transfer membrane. Membranes were prehybridized before hybridization for 24h with radiolabelled cDNA probes for rPRL (Gubbins et al, 1979) or rGH (Harpold et al, 1978), then washed to remove unbound probe, and subjected to autoradiography for 3-5 days at  $-70^{\circ}\text{C}$ . Statistical comparisons were made with one-way analysis of variance.

## 2.3 RESULTS

### 2.3.1 Dose-dependent inhibition of PRL release and mRNA levels

Both CV 205-502 and CQP 201-403 caused similar dose-dependent inhibition of PRL release in initial experiments to 8.9% and 13.6% of control release respectively at 100nM concentrations ( $P < 0.001$  compared to control; Figure 2.2). A similar dose-dependent inhibition was seen with bromocriptine, although the maximal suppression was slightly less (24.7% of control secretion at 100nM bromocriptine). GH release in these experiments was somewhat variable but no consistent dose-dependent effect was seen ( $P > 0.05$ ; Figure 2.3).

Both agents inhibited PRL mRNA accumulation. The dose-dependency of mRNA accumulation with each drug was somewhat variable, but statistically significant inhibition of PRL mRNA levels (to 69% and 52% of control in single experiments) was seen with doses of CV 205-502 and CQP 201-403 as low as 1nM ( $P < 0.05$  compared to control; Figure 2.4). However the maximal inhibition was in all experiments less than that seen for PRL release, similar to that seen with bromocriptine, and the greatest degree of suppression seen in individual experiments was to 42% of control with 100nM CQP 201-403.

### 2.3.2 Pooled data for 100nM doses of CV 205-502 and CQP 201-403

Because of individual variability between dose-response experiments, a comparison was made of maximally effective PRL-inhibitory doses of CV 205-502, CQP 201-403 and bromocriptine using data pooled from three separate experiments; this confirmed the striking inhibition achieved by all three agents on PRL release ( $P < 0.005$  compared to control in each case), slightly greater with CV and CQP than bromocriptine.

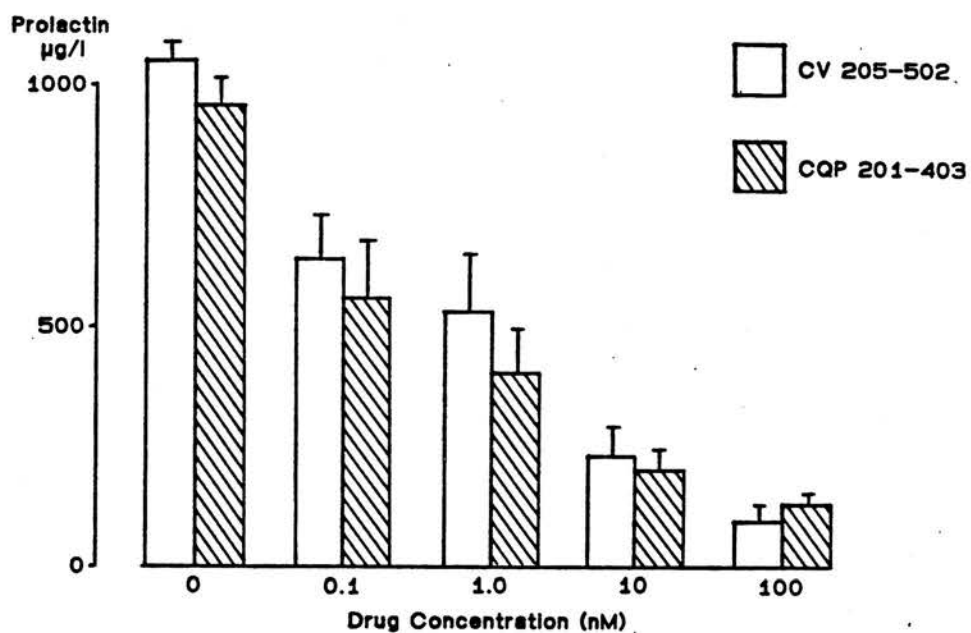


Figure 2.2 Effect of increasing doses of CV 205-502 (open bars) and CQP 201-403 (hatched bars) on PRL release from cultured rat pituitary cells. Data are means  $\pm$  SEM,  $n = 7$  replicate culture wells, pooled from two separate experiments; \*  $P < 0.001$  compared to control.

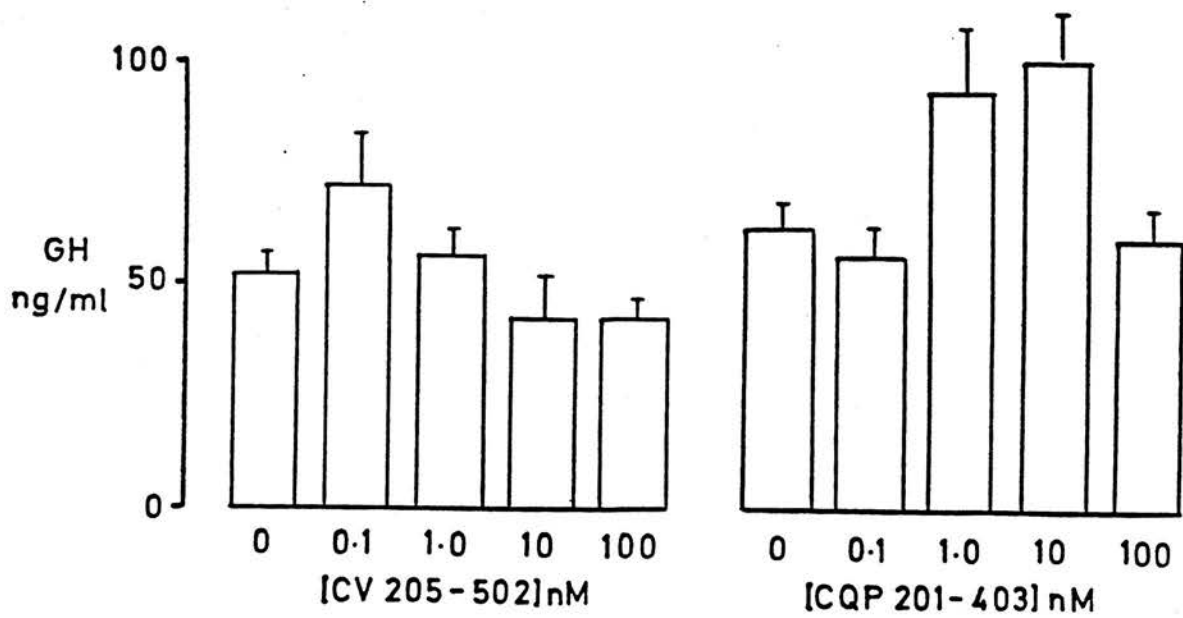


Figure 2.3 Effect of increasing doses of CV 205-502 and CQP 201-403 on GH release from cultured rat pituitary cells. Data are means  $\pm$  SEM,  $n = 7$  replicate culture wells, pooled from the two separate experiments shown in Figure 2.2; \*  $P < 0.001$  compared to control.



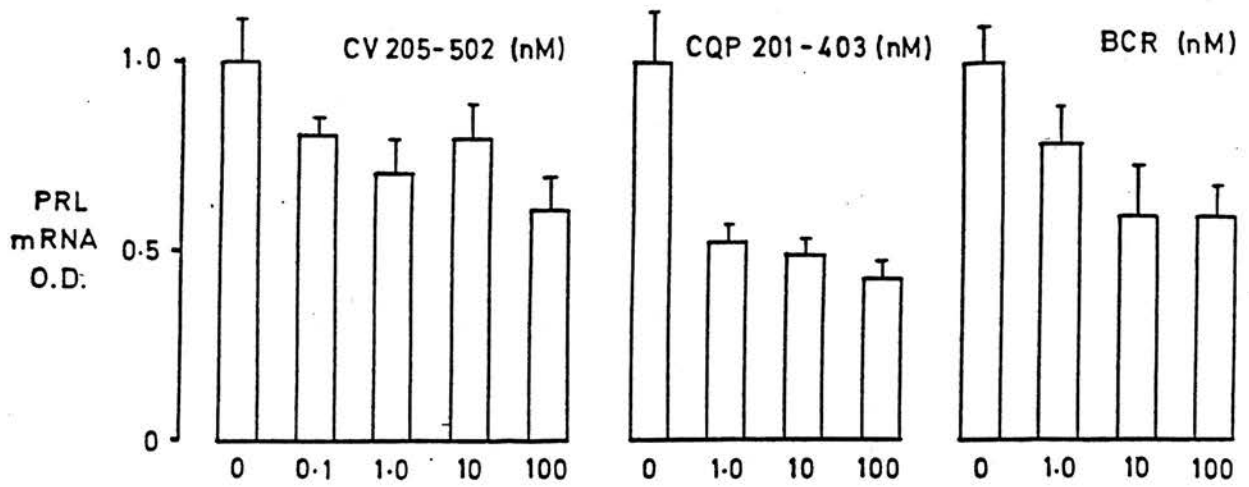


Figure 2.4 Effects of increasing doses of CV 205-502, CQP 201-403 and bromocriptine on relative PRL mRNA concentrations. mRNA data are expressed in arbitrary units of optical density (O.D.), relative to control values of 1.0. Data are means  $\pm$  SEM,  $n = 3-4$  replicate culture wells from single experiments in each case; \*  $2P < 0.05$  compared to control.

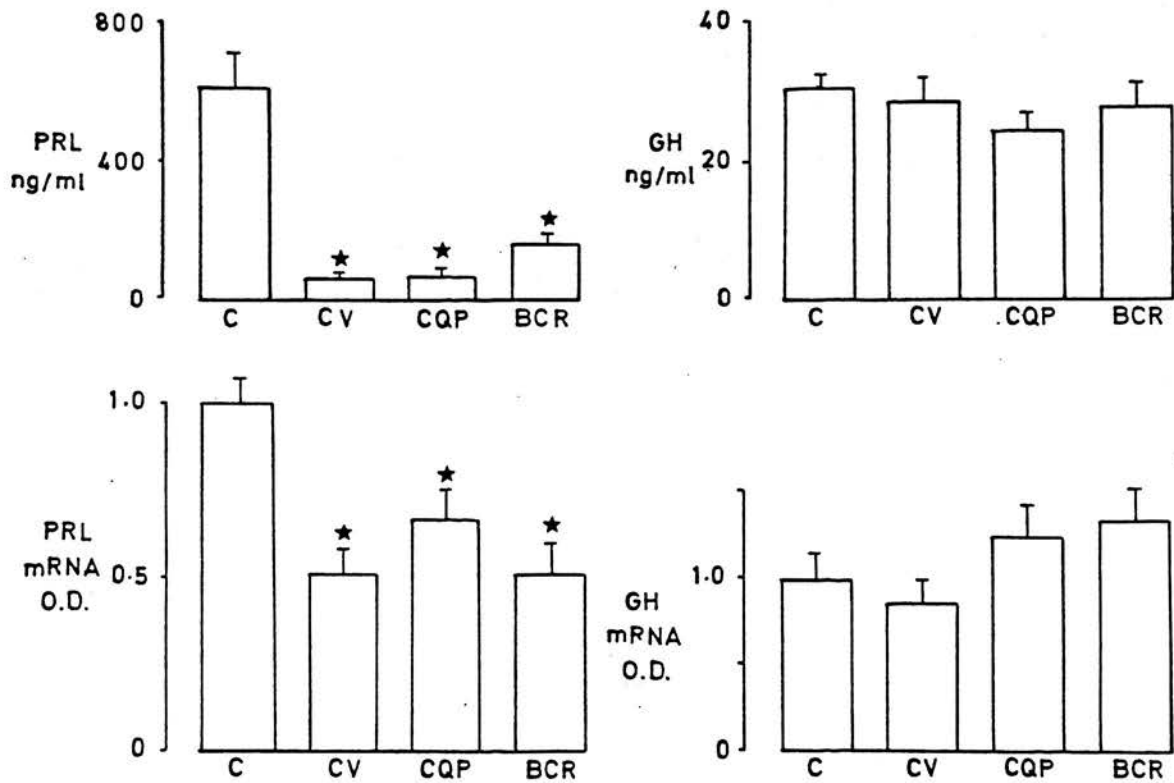


Figure 2.5 Effects of 100nM doses of CV 205-502, CQP 201-403 and bromocriptine on PRL release, PRL mRNA concentrations, GH release and GH mRNA concentrations. Messenger RNA data are expressed in arbitrary units of optical density (O.D.), relative to control values of 1.0. Data shown are combined results of 3 (PRL data) or 2 (GH data) separate experiments, n = 8-18 replicate culture wells for each group; \* 2P < 0.005 compared to control.

PRL mRNA accumulation was clearly reduced by all three dopamine agonist agents, but this inhibition was less marked than that of PRL secretion, only to 50%, 67% and 51% of control values by CV 205-502, CQP 201-403 and bromocriptine respectively ( $P < 0.005$  compared to control in each case; Figure 2.5). In contrast, no inhibitory effects were seen on either GH release or GH mRNA accumulation ( $P > 0.5$ ; Figure 2.5).

## 2.4 DISCUSSION

In this Chapter, the technique of cytoplasmic dot hybridization analysis has been applied to study dopaminergic inhibition of PRL mRNA accumulation in primary cultures of rat pituitary cells. The data confirm previous data for the inhibitory effect of bromocriptine on rPRL mRNA and show that both of the novel dopamine agonist drugs had similar inhibitory effects on PRL but not GH production.

In contrast to the profound inhibitory effects of both agents on PRL release, the inhibition of PRL mRNA accumulation was less marked, but comparable to that achieved by a similar high (100nM) dose of bromocriptine. The reason for this lesser effect on mRNA levels may be the relatively long half-life of PRL mRNA (~ 17 hours; Laverriere et al, 1983; Rosenfeld et al, 1987), which means that rapid changes in the rate of gene transcription have slow effects on the cytoplasmic accumulation of mRNA species. In previous studies of the effect of bromocriptine, 50% inhibition of PRL mRNA accumulation was seen between 24 and 48 hours, (similar to the present findings with CV 205-502 and CQP 201-403), though maximal suppression of mRNA levels took up to 96 hours (Maurer, 1980). These two newer dopamine agonist drugs are more potent than bromocriptine *in vivo* (Rasmussen et al, 1987; Hanssen et al, 1988),



yet the data shown here do not provide clear evidence for greater potency at the level of mRNA accumulation, and further studies of longer term effects of lower doses of these drugs would be necessary to detect differences between the potency.

Neither CV 205-502 nor CQP 201-403 had any significant effect on either GH release or GH mRNA levels in this study. One previous study of these drugs *in vitro* showed a small inhibition of GH release with CQP 201-403 and no effect with CV 205-502 (Venetikon et al, 1987), although both drugs at least partly blocked the stimulation of GH release by GRF. The present data confirm previous findings that GH mRNA levels were unaffected by bromocriptine (Wood et al, 1987), as was found with both the new dopamine agonist drugs.

In conclusion, these results document suppressive effects on lactotrophs of three structurally different dopamine agonist agents, both at the level of acute hormone release but also a pre-translational site of action, to reduce PRL mRNA accumulation and hence PRL synthesis. Thus the technique of cytoplasmic dot hybridization can be applied to normal rat pituitary tissue to demonstrate dopaminergic suppression of PRL mRNA levels. Chapter 3 will describe the application of the same techniques in an *in vitro* study of an aggressive prolactinoma, in an attempt to study hormonal regulation of the endogenous human PRL gene.

## CHAPTER 3

### AGGRESSIVE PROLACTINOMA:

#### CLINICAL AND CELL CULTURE STUDIES

##### 3.1 INTRODUCTION

Prolactin-secreting pituitary tumours commonly present in women as small intrasellar microadenomas, but especially in men, the tumours may be much larger, and present as macroadenomas more than 1cm in diameter (Nabarro, 1982; Grossman & Besser, 1985). Dopamine agonist drugs have an important role in the management of these tumours, and may be highly successful in reducing their size as well as their endocrine effects (Vance & Thorner, 1987). "Giant" prolactinomas, of several centimetres diameter and with major extrasellar tumour extension, are rare and may provide special problems of management. One reported case showed marked shrinkage within six days of bromocriptine therapy, and almost complete regression of the tumour mass after 6 months of treatment (Clayton et al, 1985). Two further cases of giant invasive prolactinomas (more than 4cm diameter) treated with bromocriptine have been reported (Murphy et al, 1987): one tumour was only partially responsive to bromocriptine, both in terms of serum PRL levels and tumour size, although the other tumour had responded satisfactorily by about eight years after radiotherapy.

There have been a limited number of *in vitro* studies on human PRL-secreting tumours, usually using perifusion systems, (for example, Venetickou et al, 1987; Bevan et al, 1989). These studies have provided useful information on the hormonal responsiveness of these tumours in terms of PRL secretion *in vitro*, which may differ from that found in normal rat or human pituitary tissue (Jones et al, 1989). The properties of the type

2 dopamine receptor appear to be essentially normal in non-functioning tumours (Bevan et al, 1989), but in prolactinomas responses to dopamine or bromocriptine *in vitro* appear to differ among different tumours (Bevan et al, 1989).

There is so far no information about prolactin gene expression in human prolactinomas. One reason for this is the limited amount of tissue available for *in vitro* study: in many centres the referral rates for pituitary surgery in these patients are relatively low compared to other tumour types, patients being treated with dopamine agonists alone or in conjunction with radiotherapy. One study has recently reported that the *v-fos* protooncogene was amplified in a single prolactinoma (U et al; 1988), but there have so far been no reports of regulated expression of the hPRL gene itself.

In the last chapter, rPRL mRNA levels were shown to be readily suppressible by three different dopamine agonist drugs, suggesting that the technique of cytoplasmic dot hybridization would be appropriate to attempt to study the endogenous hPRL gene in prolactinoma tissue. In this chapter one case of an unusually aggressive and locally invasive prolactinoma will be described, with the results of cell culture studies that were performed on the tumour to assess regulation of PRL secretion and mRNA levels by bromocriptine.

### 3.2 CASE REPORT

The patient (M.H.) first presented in 1982 at the age of 40y with a 6 week history of frontal headache, diplopia and right sided ptosis. He had been previously fit and healthy, and took no medication. On examination, he had partial right 3rd, 4th and 6th cranial nerve palsies, and there was

partial loss of the nasal field of vision in the left eye. Skull X-ray showed an expanded pituitary fossa, and CT scan revealed a tumour extending laterally and into the right sphenoid sinus, but with little suprasellar extension. Endocrine testing pre-operatively revealed a serum PRL of 91,600 mU/L (normal levels <500mU/L), and partial hypopituitarism. At a frontal craniotomy all of the intrasellar tumour was removed, but some of the laterally extending tissue was not accessible. Histological examination of the pituitary tissue revealed "excessive nuclear variation" with a large number of mitoses.

Post-operatively, serum PRL levels fell only slightly but he became panhypopituitary. He was treated with hydrocortisone, thyroxine and testosterone replacement therapy, and commenced on bromocriptine 7.5 mg daily, before receiving 4500cGy external irradiation of the pituitary region (three fields, 30 fractions over six weeks). Serum PRL levels fell to a nadir of 11,000 mU/L after two months on bromocriptine 7.5 mg daily, but over the next nine months through 1983 it gradually rose to over 39,000 mU/L, despite increasing doses of the drug to 15 mg daily (Figure 3.1).

Bromocriptine was discontinued during 1984 in view of its lack of sustained effect on serum PRL levels, but he remained clinically well over the next two years. However, in April 1986, he developed right sided 9th, 10th and 12th cranial nerve palsies, in addition to his pre-existing 3rd, 4th and 6th nerve palsies. Serum PRL was 120,000 mU/L (Figure 3.2). CT scan appearances were little changed compared to 1982, but the tumour was assumed to have extended inferiorly to cause lower cranial nerve damage. A further trial of bromocriptine was commenced, using doses up to 30 mg daily. Although this indeed reduced PRL levels to 20,000 mU/L (Figure 3.2), the patient experienced severe nausea, and in June 1986 he underwent

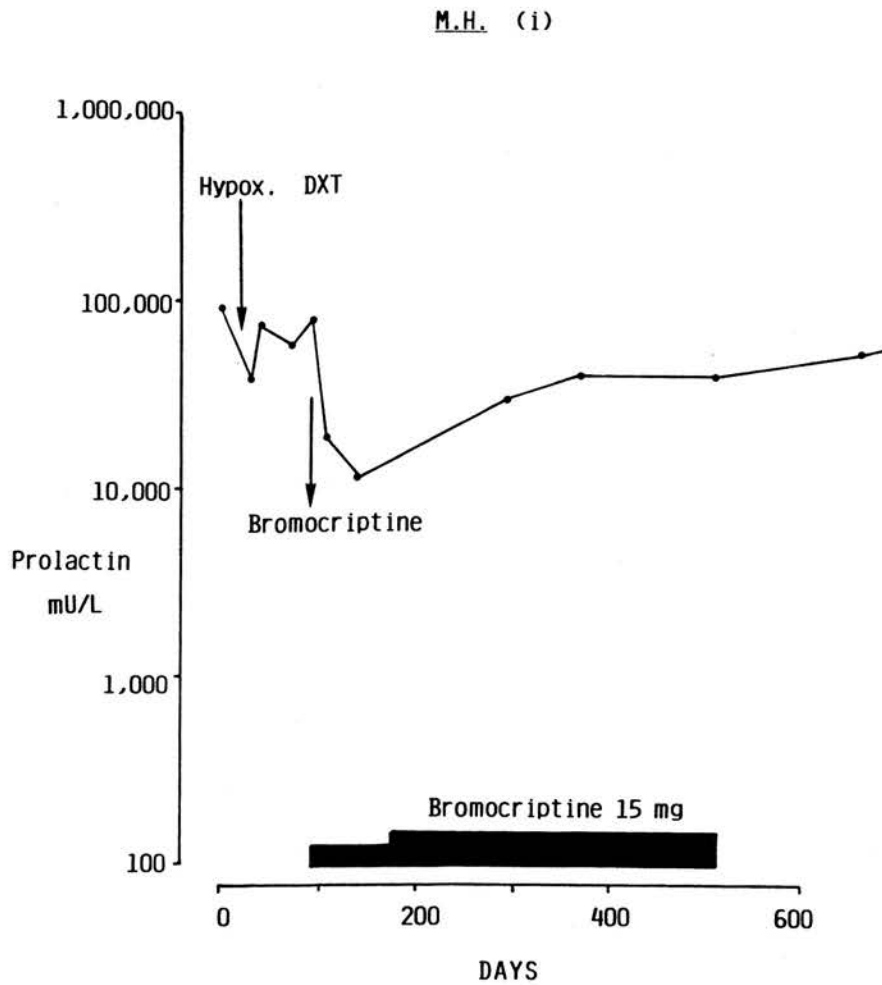


Figure 3.1

Patient MH: serum PRL levels 1982-1984 (days 0-600), showing the first hypophysectomy (Hypox) and radiotherapy (DXT), and the initial treatment with bromocriptine (dose indicated by hatched bar). Note logarithmic vertical scale.



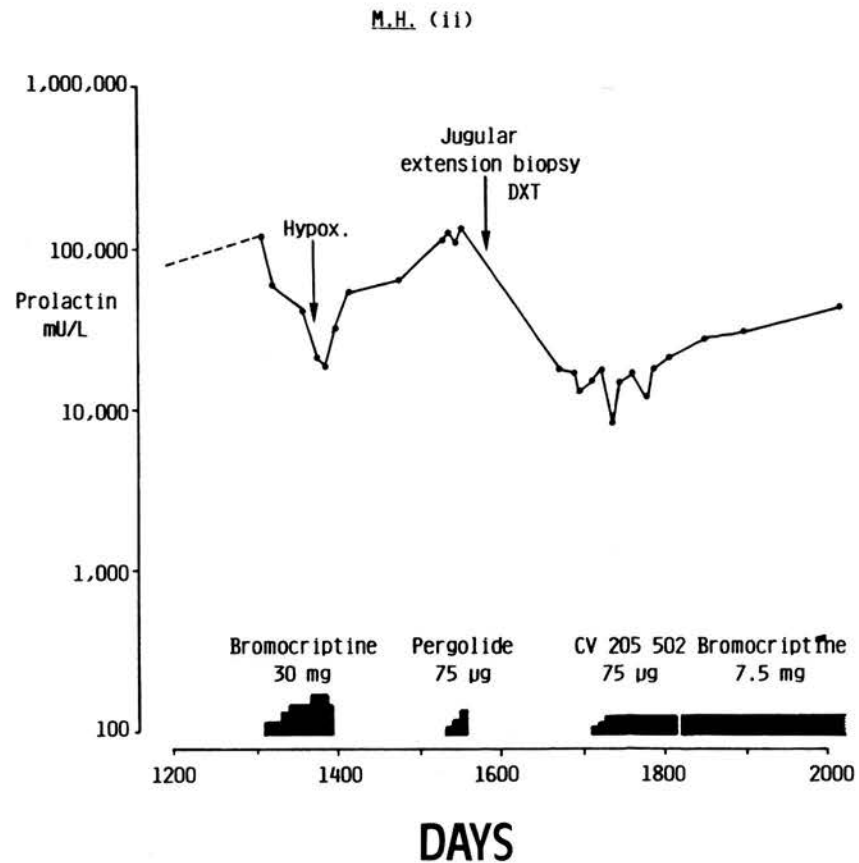


Figure 3.2

Patient MH: serum PRL levels 1986-88 (days 1200-2000), showing the second hypophysectomy (Hypox), and radiotherapy (DXT) to the skull base, and further treatment with different dopamine agonist drugs (doses indicated by hatched bars). Note logarithmic vertical scale.

a transethmoidal hypophysectomy in an attempt to reduce the tumour bulk. A large mass of tumour extended laterally to the right, superiorly and inferiorly, and was largely removed. Histological examination showed a cellular chromophobe neoplasm with solid sheets of pleomorphic cells with hyperchromatic nuclei and a variable amount of cytoplasm. Numerous binucleate cells and mitotic figures were found. Tumour tissue was obtained at this operation for the *in vitro* studies of PRL release and mRNA accumulation.

Serum PRL levels remained very high, despite a trial of pergolide treatment. However the patient then developed a right parapharyngeal abscess. This was treated successfully with antibiotics, but a residual hard lymph node was found at the angle of the right mandible. Excision biopsy revealed a lymph node adjacent to a mass of tumour tissue within the internal jugular vein. Histological examination revealed only reactive changes in the lymph node, but confirmed infiltration of the vein wall with pleomorphic cells which showed positive immunostaining for PRL; furthermore a fragment of this tissue secreted immunoreactive PRL when cultured *in vitro* (data not shown), confirming extension of the pituitary tumour.

The patient was treated with further radiotherapy to the neck and skull base (3000cGy, 10 fractions over 14 days), in an effort to halt further extracranial extension of the pituitary tumour, and serum PRL levels in fact fell markedly over the next three months from 140,000 to 18,000 mU/L, in the absence of any further bromocriptine therapy. A subsequent trial of the dopamine agonist drug CV 205-502 (described in Chapter 2) was started in an attempt to reduce tumour bulk in the inferior petrosal sinus. Acute administration of this agent caused transient suppression of serum PRL within 24 hours, similar to that seen with bromocriptine (Figure 3.3).

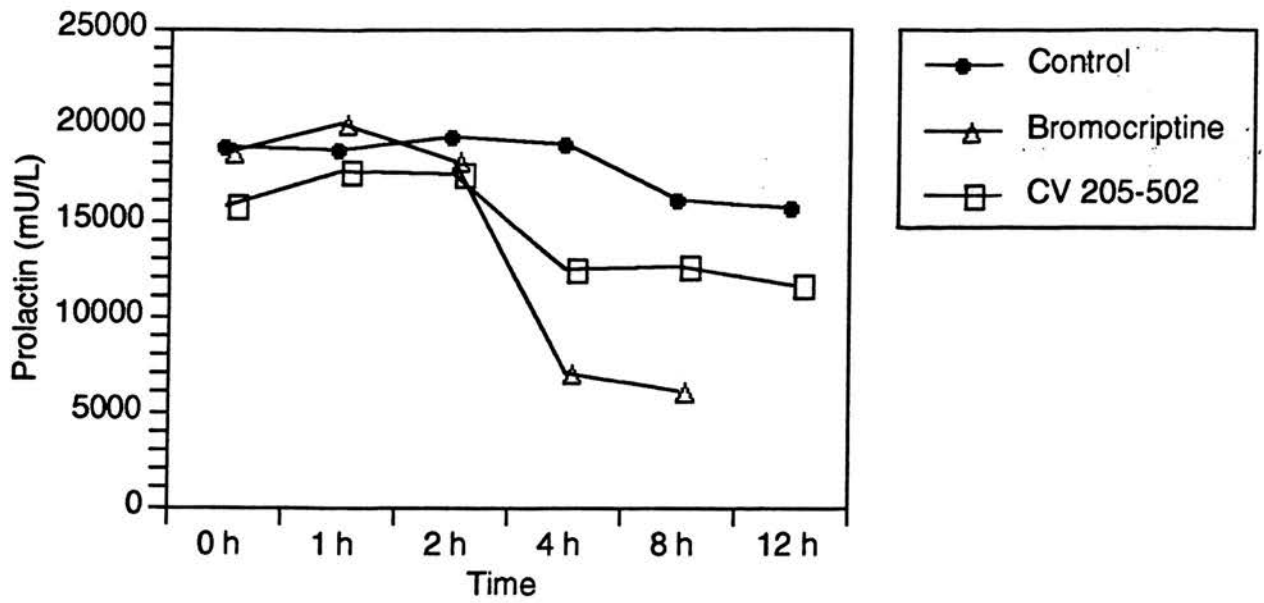


Figure 3.3

Patient MH: response of serum PRL to single doses of bromocriptine (2.5mg; open triangles) or CV205-502 (25µg; open squares). Serum PRL levels during a control day are shown by a solid line. Serum PRL is shown here on a linear scale for clarity.

However this suppression was not sustained with chronic treatment with CV 205-502 (75 µg daily) over the subsequent three months, and serum PRL remained between 12,600 and 22,100 mU/L. The patient reverted to bromocriptine treatment, but PRL levels continued to rise gradually to over 46,000 mU/L in 1988. The patient complained of facial pain at this time, and clinical examination suggested involvement of the right fifth cranial nerve by tumour. A third hypophysectomy operation was performed in a further attempt to reduce the tumour bulk. Tumour tissue was obtained at the second hypophysectomy operation (1986) for the studies described in this Chapter, and from the third operation (1988) for the transfection study described in Chapter 7.

### 3.3 CELL CULTURE STUDIES

#### 3.3.1 Methods

Pituitary tumour tissue was enzymatically dispersed using collagenase and hyaluronidase, using the same techniques employed for rat pituitary tissue (see Chapter 2 and Appendix A1.1). The yield of pituitary cells was approximately  $9.0 \times 10^6$  cells, with a viability of 85% as assessed by trypan blue dye exclusion. Dispersed prolactinoma cells were plated onto plastic cell culture dishes at a density of approximately  $2 \times 10^5$  viable cells in 2ml per well in final culture medium (Dulbecco's modified Eagle's medium containing 10% horse serum, 2.5% fetal calf serum and 2mM glutamine). Cells were allowed to attach to the plastic dishes and were grown in monolayer culture for five days before experiments commenced; they were then washed by repeated medium exchanges, and treated with bromocriptine or dopamine in serum-free medium for 24 hours.

Bromocriptine (Sigma Chemical Co, Poole, Dorset) was dissolved initially

in ethanol before dilution in culture fluid; dopamine (Sigma) was dissolved in culture fluid with ascorbic acid, in order to reduce degradation of the dopamine. Thyrotrophin-releasing hormone (TRH; Roche Pharmaceuticals, Welwyn, Herts) was dissolved directly in culture fluid.

Medium was then removed for radioimmunoassay of released hormone, using MRC 83/562 as hPRL standard, and MRC 66/217 as hGH standard. These radioimmunoassays were kindly performed by Miss Judy Webb and Dr DA Heath, Department of Medicine, Queen Elizabeth Hospital (PRL), and Dr SS Lynch, Department of Clinical Endocrinology, Birmingham & Midland Hospital for Women (GH). Blank samples consisting of culture fluid alone showed no cross-reaction in either assay, and assay coefficients of variation were less than 10%.

Cytoplasmic extracts were prepared for dot hybridization analysis, which was carried out as described for the studies in Chapter 2 (Appendix 3.1), using a  $^{32}\text{P}$ -labelled cDNA probe specific for hPRL (Truong et al, 1984), which was generously provided by Dr JA Martial, Université de Liège, Belgium. The DNA probe was radiolabelled by nick translation, as described in Appendix 2.1.

### 3.3.2 Results

Over the first four days in culture, the cells produced large quantities of PRL, and very small amounts of GH (Figure 3.4); however GH secretion was not detectable in subsequent 24h incubations, implying that any contamination with normal pituitary tissue was negligible. Treatment for 24h with both 10nM and 100nM bromocriptine reduced PRL release from the cultured cells to approximately 50% of control values; 10 $\mu\text{M}$  dopamine had a similar effect. On the other hand, hPRL mRNA levels were only minimally reduced by bromocriptine, and the single high dose of dopamine had no

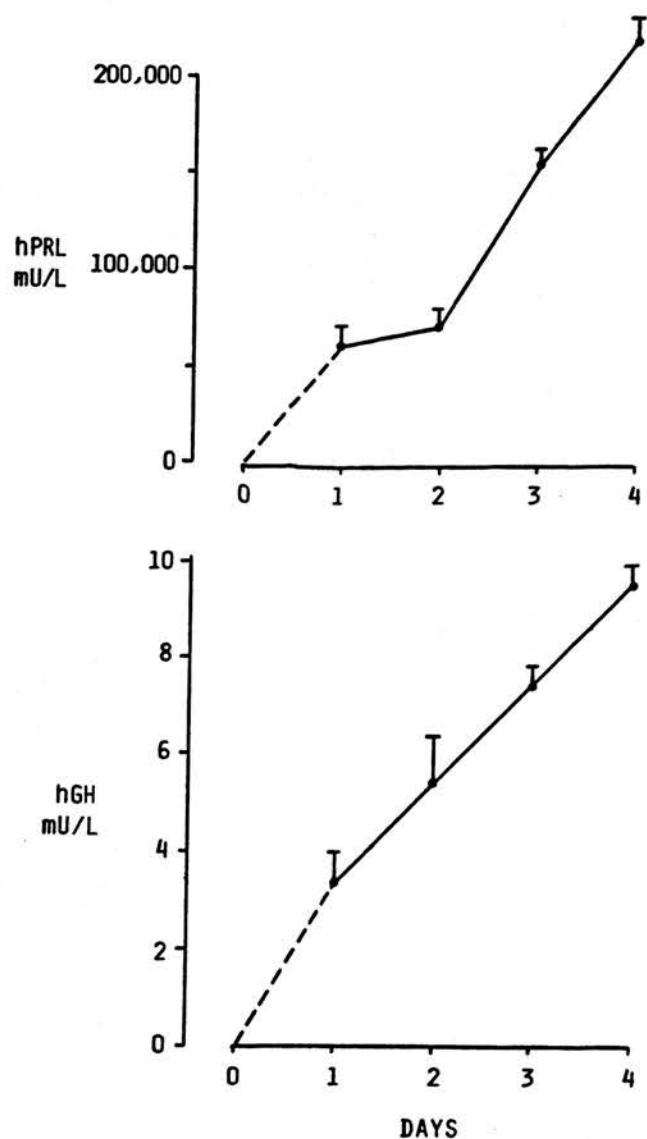


Figure 3.4

Cultured human prolactinoma cells: accumulation of PRL and GH in culture fluid over days 1-4. Data are means  $\pm$  SEM, n=4 replicates in each case.

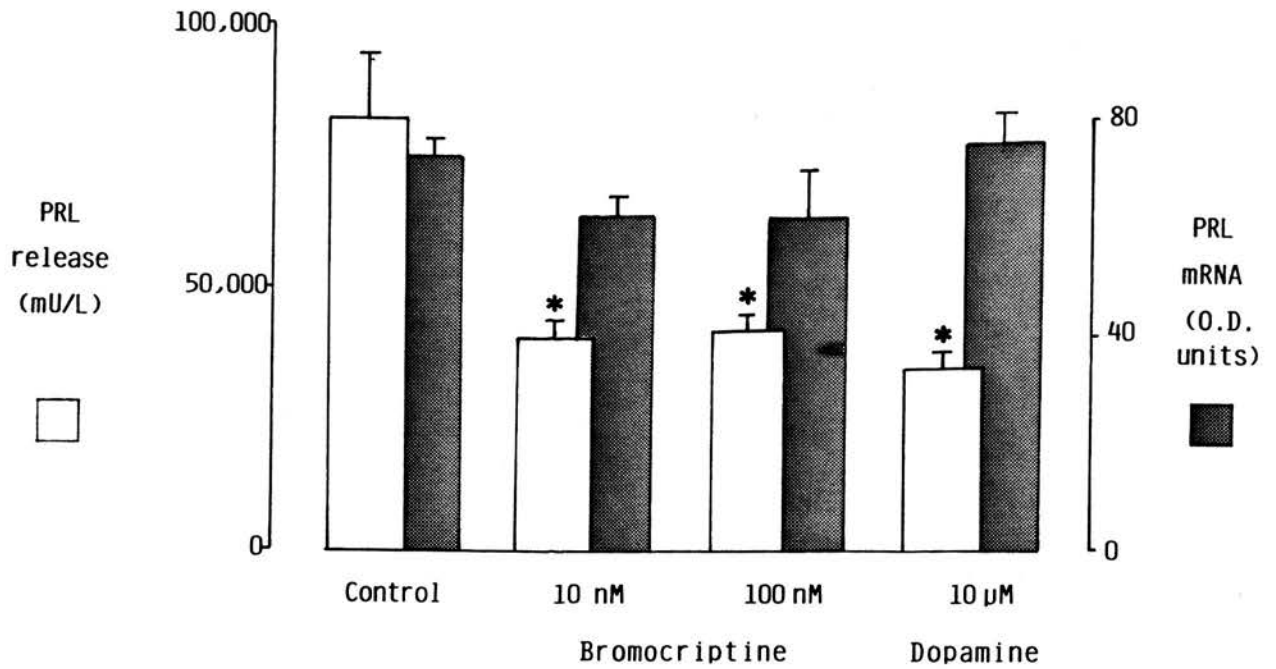


Figure 3.5

Cultured prolactinoma cells: PRL secretion (open bars) and mRNA accumulation (stippled bars). Cells were treated for 24h as shown, and mRNA data are expressed in arbitrary units of optical density. Data are means  $\pm$  SEM, n=4 per group; \*P<0.05, \*\*P<0.002 compared to control.

effect (Figure 3.5). These results contrast markedly with the data presented in Chapter 2 (and see also Wood et al, 1987) using normal rat pituitary tissue, where dopaminergic inhibition of rPRL mRNA levels to about 50% of control was readily observed.

A single high dose of TRH (100nmol/L) stimulated PRL release, but had only marginal and non-significant effects on hPRL mRNA levels (not shown), again in contrast to its well documented effects in rat pituitary tissue.

### 3.4 DISCUSSION

The case presented in this Chapter had an exceptionally aggressive and locally invasive PRL-secreting pituitary tumour, which had spread along the skull base with local extension through the inferior petrosal sinus to emerge from the skull within the internal jugular vein. The tumour was partially responsive to dopamine agonist therapy, because acute challenges with bromocriptine and CV 205-502 transiently suppressed serum PRL levels. However the suppression of serum PRL was not sustained despite moderately high doses of three different dopamine agonist drugs. Resistance of a small proportion of prolactinomas to dopamine agonist therapy is well-recognised (Thorner et al, 1980; Breidahl et al, 1983; Bannister & Sheridan, 1987), but the reasons for this resistance are not understood. Although this is just a single and exceptional case, the opportunities to study such tumours *in vitro* are rare and may provide valuable information about the potentially abnormal intracellular regulation of hormone production.

The cellular basis of for this dopamine resistance is not yet understood. In a series of human pituitary tumours, membrane-associated dopamine (D-2) receptors appear to be present and to have normal binding characteristics



(Bevan et al, 1986). In two dopamine-resistant lactotroph tumours in the rat, loss of membrane receptor-associated G-proteins has been described (Collu et al, 1988), which would explain a loss of dopamine action despite intact dopamine-receptor binding. The present tumour did respond to dopamine agonists *in vivo*, and also *in vitro* in terms of hormone secretion, so lack of dopaminergic receptors or absence of receptor-associated proteins cannot explain the longer-term dopamine "resistance". However there was no significant effect of high doses of either bromocriptine or dopamine on hPRL mRNA accumulation. This contrasts with the findings in normal rat pituitary tissue presented in Chapter 2, where bromocriptine and two other dopamine agonists caused readily detectable suppression of rPRL mRNA levels (albeit to a lesser extent than hormone secretion). The techniques used for these experiments were identical, so it seems unlikely that this reflects a technical limitation of the hybridization assay.

In the present tumour, therefore, it appears that hPRL mRNA accumulation (and hence, presumably, hPRL synthesis) is relatively independent of regulation by TRH and dopamine, in contrast to short-term secretion of the hormone. If abnormalities at the level of membrane receptors and G-proteins are excluded by the PRL secretion data, then these findings suggest a possible abnormality between second messenger generation and gene transcription. Examples of such a mechanism might include abnormal regulation or production of a protein kinase or its substrate, or a nuclear DNA-binding protein. Amplified expression of certain proto-oncogenes in pituitary tumours could have these effects. Evidence that such a supposition may be correct has come from U et al (1988) who analysed genomic DNA from a single prolactinoma and a normal pituitary gland, and found amplification of *v-fos* in the tumour. The *fos* proto-oncogene is

known to be involved in the action of AP-1 (Curran & Franza, 1988), and is responsive to cyclic AMP (Billestrup et al, 1987), and therefore amplified *v-fos* expression could conceivably result in sustained non-suppressible stimulation of PRL gene transcription, without necessarily interfering in the hormonal regulation of acute hormone release.

In order to be more confident of such speculative conclusions it will be necessary to study more pituitary tumours to see if the present findings are typical or exceptional. While prolactinomas are now only rarely available for *in vitro* study in many centres, GH-secreting tumours are more readily obtainable from pituitary surgery, and data for hGH mRNA regulation in a series of such tumours will be presented in Chapter 4.

## CHAPTER 4

### GROWTH HORMONE mRNA REGULATION IN HUMAN SOMATOTROPH TUMOURS

#### 4.1 INTRODUCTION

Knowledge of the hormonal control of GH gene expression has so far been largely based on studies of rat pituitary cells, and the availability of clonal rat pituitary cell lines has allowed detailed examination of the mechanisms involved as outlined in Chapter 1. Detailed study of the endogenous human GH (hGH) gene has been limited by the difficulty in obtaining sufficient tissue, but may be important in relation to the possible pathogenesis of GH-secreting pituitary tumours. Cytoplasmic levels of hGH mRNA in pituitary tumour cells may be suppressed *in vitro* by insulin-like growth factor 1 (IGF-1; Yamashita et al, 1986) and increased by dexamethasone but not by thyroid hormone in contrast to rat GH (rGH) mRNA (Isaacs et al, 1988). However there is so far no information as to the possible effects of the other important regulators of somatotroph function on endogenous hGH gene expression in human pituitary tissue.

In the preceding Chapter, a single aggressive prolactinoma was studied using the technique of cytoplasmic dot hybridization, and tentative conclusions were drawn from the apparent resistance of mRNA levels to hormonal manipulation *in vitro*. GH-secreting tumours are frequently treated primarily by pituitary surgery, and therefore this tissue is more readily available for *in vitro* study. In view of this, it was decided to apply the techniques used in the earlier study to a series of GH-secreting tumours obtained from hypophysectomy operations carried out at the Queen

Elizabeth Hospital, Birmingham. In this Chapter data is presented on the hormonal and intracellular control of hGH release and mRNA accumulation in cultured cells from six human somatotrophic tumours. The cells were exposed to various substances including a GH-releasing factor fragment (GRF 1-29 amide), somatostatin and bromocriptine; in addition forskolin and phorbol ester were tested in order to determine the possible involvement of adenylate cyclase and protein kinase C in hGH synthesis and secretion.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Cell culture

Pituitary adenoma tissue was obtained for cell culture from routine transsphenoidal or transfrontal hypophysectomy operations on six patients with acromegaly. Only one of the tumours (tumour 1, patient EJ) was markedly aggressive and resistant to therapy, with very high GH levels and major extrasellar extension. He had had two previous hypophysectomy operations, with radiotherapy twelve years earlier; he had failed to respond to bromocriptine, and had only a partial response to somatostatin (GH levels falling from 6700 to 1900 mU/L. The tissue was obtained from this man's third hypophysectomy. The other tumours were more typical, and the pituitary surgery was the primary mode of therapy; patient details are outlined in Table 4.1.

At operation, pituitary tumour tissue was immediately placed in ice-cold culture fluid, which consisted of Dulbecco's modified Eagle's medium (Gibco BRL, Paisley, Scotland), supplemented with 10% horse serum and 2.5% fetal calf serum (Gibco BRL) with antibiotics. The tissue was chopped into small

Tumour number	Patient's sex & age	Basal serum GH pre-operatively (mU/L)	Unstimulated GH release <i>in vitro</i> (mU/L; mean $\pm$ SEM)
1	M, 34y	4100	75 $\pm$ 10
2	M, 42y	32	67 $\pm$ 10
3	M, 42y	160	249 $\pm$ 39
4	M, 39y	130	398 $\pm$ 30
5	F, 42y	130	64 $\pm$ 4.7
6	F, 36y	14	55 $\pm$ 2.5

Table 4.1

Summary of patient details, showing random pre-operative serum GH levels, with mean unstimulated GH release *in vitro* over a 24h incubation period (these data are means  $\pm$  SEM, n=3-8 replicates in each case).

fragments and enzymatically dispersed using collagenase and hyaluronidase as for the studies in Chapters 2 and 3 (described in Appendix A1.1).

Each pituitary tumour yielded between  $2.0$  and  $7.8 \times 10^6$  cells, with a viability determined by trypan blue exclusion of 84-95%. Dispersed adenoma cells were plated onto plastic culture dishes at a density of  $1-2 \times 10^5$  cells per well in 2ml of culture fluid, incubated at  $37^\circ\text{C}$  in a humidified atmosphere of 5%  $\text{CO}_2$ : 95% air, and allowed to grow in monolayer culture for at least five days before experiments were carried out. The cells were then washed by repeated (serum-free) medium exchanges and treated for 24h with test substances in serum-free culture fluid. After treatment periods culture fluid was removed for radioimmunoassay of released hGH, and cell cytoplasmic extracts were prepared for mRNA determinations.

#### 4.2.2 Materials and radioimmunoassays

GRF 1-29 amide (of equal potency to authentic human GRF; Bachem, Saffron Walden, Essex, UK) and somatostatin 1-14 (Sigma Chemical Company, Poole, Dorset, UK) were dissolved directly into culture fluid. Forskolin (Calbiochem, Cambridge, UK) and bromocriptine (Sigma) were dissolved initially in ethanol, and the phorbol esters 12-O-tetradecanoyl phorbol-13-acetate (TPA; Sigma) and phorbol 12,13-didecanoate (PDD; Sigma) were dissolved in dimethylsulphoxide, before dilution in culture fluid immediately before experiments.

Human GH released into culture fluid was measured by radioimmunoassay using MRC 66/217 hGH standard; these assays were kindly carried out by Dr SS Lynch, Birmingham & Midland Hospital for Women. Blank samples consisting of culture fluid alone did not cross-react in this assay. Mid-range intra- and inter-assay coefficients of variation were less than 10%.

#### 4.2.3 mRNA measurements

Messenger RNA accumulation was measured by cytoplasmic dot hybridization as in Chapters 2 and 3 (described in Appendix A3.1). Cells were scraped from culture dishes and cytoplasmic extracts were prepared. Cytoplasmic samples were applied in duplicate to a hybridization transfer membrane, before pre-hybridization, hybridization and autoradiography. A cDNA probe specific for hGH (Martial et al, 1979) was radiolabelled with  $\alpha^{32}\text{P}$ -dCTP using the random primer (oligo-labelling) technique (see Appendix A2.2) to a specific activity of approximately  $0.5 \times 10^9$  dpm/ $\mu\text{g}$  DNA.

The degree of hybridization was assessed by scanning densitometry of developed autoradiographs; for each experiment mRNA results were expressed in arbitrary units of optical density relative to control samples which were assigned the value of 1.0 units (100%).

For the Northern blot analysis total RNA was extracted from cells grown in monolayer culture from tumour 1 (Chirgwin et al, 1979; and see Appendix A3.2.1), and then separated by agarose gel electrophoresis before blotting onto nitrocellulose and hybridization (see Appendix A3.2.2).

#### 4.3 RESULTS

Similar densities of viable cells were plated onto culture wells in each experiment, and the amount of hGH released by unstimulated tumour cells in culture was relatively similar except for tumours 3 and 4, where basal hGH release was over four-fold higher (Table 4.1).

Cultured cells obtained from tumour 1 were used to prepare RNA for Northern blot analysis (Figure 4.1). This confirmed that a single species of hGH mRNA was present, and that its size was approximately 1000 bases, the expected size for this mRNA (Robins et al, 1982) and in accordance with

one previous report of such studies on human pituitary tissue (Yamashita et al, 1986).

The effects of 10nM GRF 1-29 amide were studied in four tumours (1,2,3 & 6; Figure 4.2); hGH release was stimulated in three of the four tumours, though the degree of stimulation varied from 129% to 199% of control values. Moderate stimulation of hGH mRNA levels was likewise seen only in these three tumours, with values of 130% to 167% of the control levels in each case.

Somatostatin (1 $\mu$ M) was studied in all six tumour cultures (Figure 4.3). GH release was inhibited in four cases, though the degree of inhibition in the responsive tumours varied from 35% to 79% of control values. In contrast, hGH mRNA accumulation was not consistently affected: slight and non-significant reductions were seen in two of the six cases, the maximum suppression being only to 70% of control values, and other values ranging between 80 and 176% of control (NS).

Bromocriptine (100nM) was studied in four tumour cultures (1,2,3 & 4; Figure 4.4). GH release was reduced in all four cases to between 50 and 75% of control values (though significantly so in only two) whereas hGH mRNA was significantly suppressed in only one of the four tumours to 70% of control, and was unaffected in the others.

All six tumour cultures were treated with 10 $\mu$ M forskolin (to activate adenylate cyclase, and thus raise intracellular concentrations of cyclic AMP), and the phorbol ester TPA (100nM; activating protein kinase C), and the results are shown in Table 4.2 (and see also Figure 4.1). Forskolin doubled hGH release and moderately increased mRNA levels in tumours 1 and 2, but had no effect in the others. However it is noteworthy that tumours 1 and 2 were also those that responded most clearly to GRF, whereas tumour



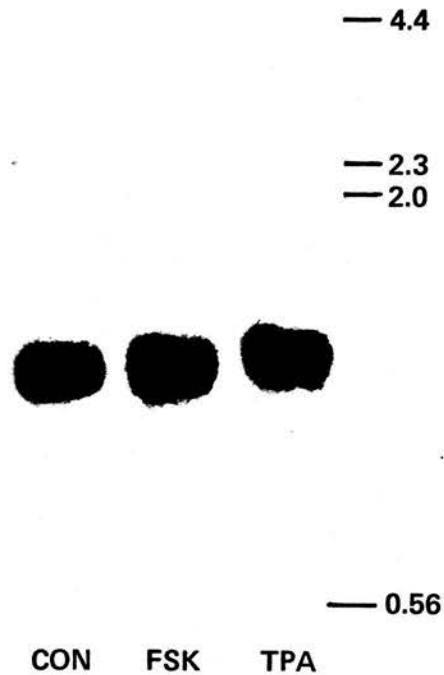


Figure 4.1

Northern blot hybridization analysis of RNA (10µg/lane) extracted from cultured cells from tumour 1. The cells were treated for 24h with control medium (CON), 10µM forskolin (FSK) or 100nM TPA before extraction of RNA. The RNA was electrophoresed on a 1% agarose gel, and blotted onto a nitrocellulose membrane for hybridization with the <sup>32</sup>P-labelled hGH cDNA probe followed by autoradiography. Standard DNA size markers (in kilobases) migrated as indicated at the right of the autoradiograph. (See also Table 2 for quantitative data from dot hybridization studies.)

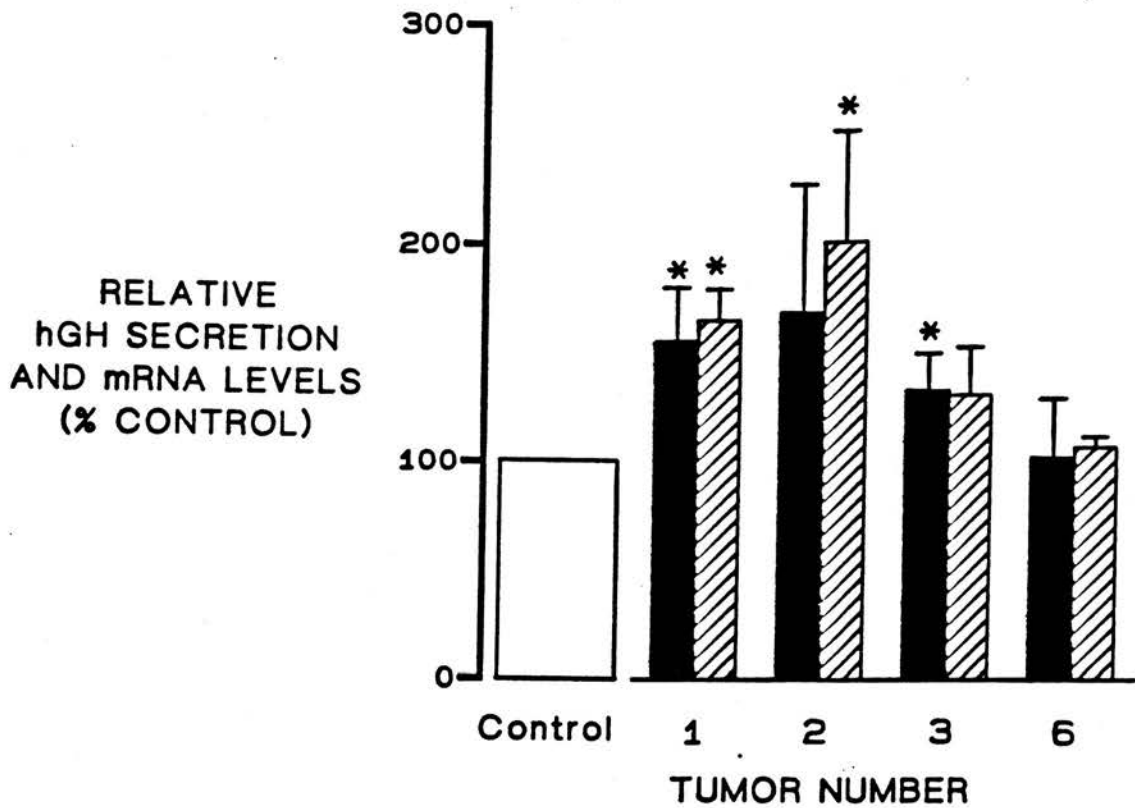


Figure 4.2

Effects of GRF on tumours 1, 2, 3 & 6. Cultured pituitary tumour cells were incubated with control medium or 10nM GRF 1-29 amide for 24h. Hormone release data (hatched bars) are expressed in terms of percentage of unstimulated hGH secretion (see Table 4.1); mRNA data (solid bars) are expressed in arbitrary units of optical density, also as percentage of control values in unstimulated groups of cells. Data are means  $\pm$  S.E.M.,  $n = 5-8$  replicate culture wells per group,  $*P < 0.02$  compared to control samples for each tumour.

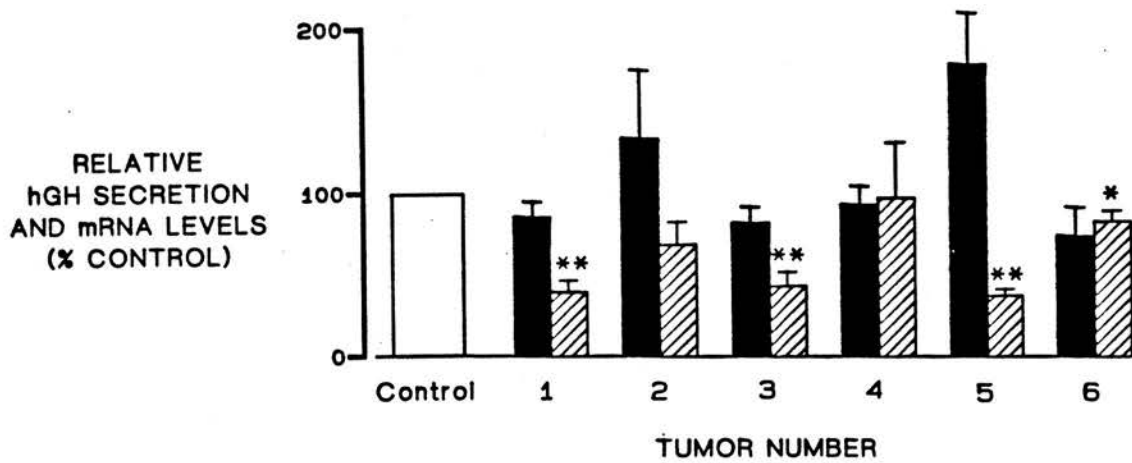


Figure 4.3

Effects of somatostatin on tumours 1-6. Cultured pituitary tumour cells were incubated with control medium or  $1\mu\text{M}$  somatostatin for 24h. Hormone release data (hatched bars) and mRNA data (solid bars) are expressed as in Figure 4.2, and are means  $\pm$  S.E.M.,  $n = 4-8$  replicate culture wells per group,  $*P < 0.02$ ,  $**P < 0.005$  compared to control samples for each tumour.

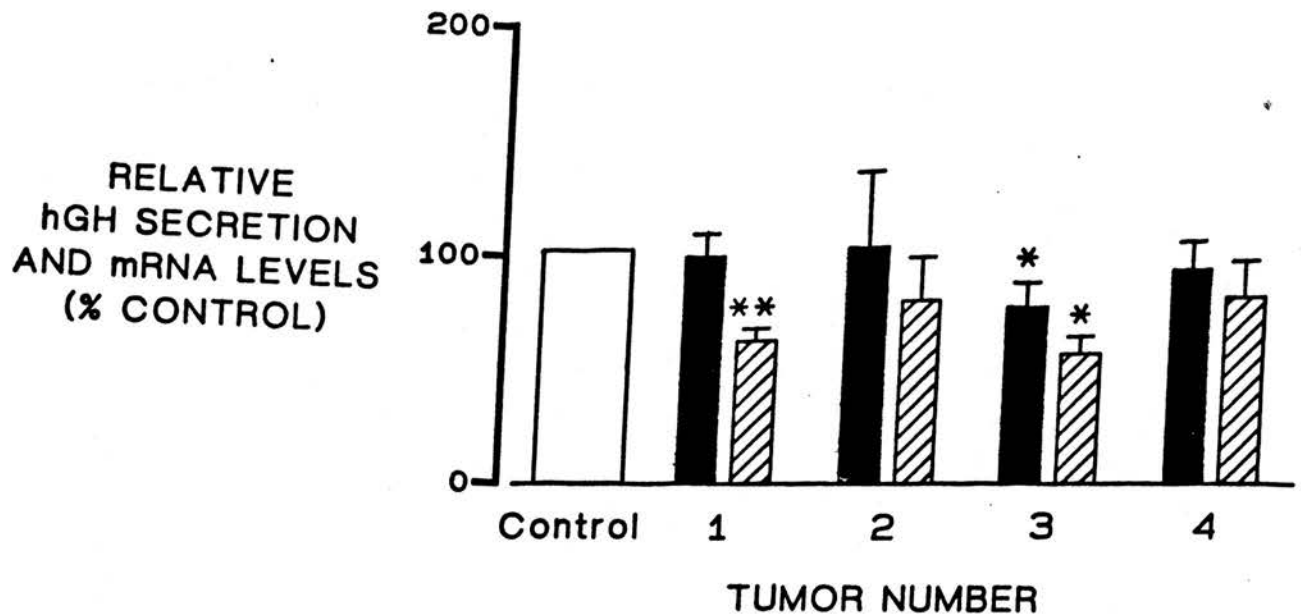


Figure 4.4

Effects of bromocriptine on tumours 1-4. Cultured cells were incubated with control medium or 100nM bromocriptine for 24h. Hormone release data (hatched bars) and mRNA data (solid bars) are expressed as in Figure 4.2, and are means  $\pm$  S.E.M.,  $n = 4-7$  replicate culture wells per group, \* $P < 0.05$ , \*\* $P < 0.005$  compared to control samples for each tumour.

	Tumour number					
	1	2	3	4	5	6
<b><u>Forskolin</u> (10<math>\mu</math>M)</b>						
hGH release (% control)	197 $\pm$ 37*	193 $\pm$ 24*	148 $\pm$ 18	77 $\pm$ 37	82 $\pm$ 5	108 $\pm$ 11
hGH mRNA (% control)	178 $\pm$ 9*	132 $\pm$ 30	80 $\pm$ 10	93 $\pm$ 44	120 $\pm$ 20	86 $\pm$ 11
<b><u>TPA</u> (100nM)</b>						
hGH release (% control)	426 $\pm$ 70**	263 $\pm$ 39**	193 $\pm$ 25*	55 $\pm$ 8*	120 $\pm$ 6	130 $\pm$ 2*
hGH mRNA (% control)	109 $\pm$ 20	155 $\pm$ 25	90 $\pm$ 10	80 $\pm$ 30	160 $\pm$ 30	112 $\pm$ 16

**Table 4.2**

GH release and mRNA levels in tumours 1-6 in response to treatment for 24h with forskolin or phorbol ester (TPA). Hormone release data are expressed in terms of percentage of unstimulated hGH secretion (see Table 4.1); mRNA data are expressed in arbitrary units of optical density, also in terms of percentage of control values in unstimulated groups of cells (100%). Data are means  $\pm$  S.E.M., n = 3-6 replicate culture wells per group, \*P<0.02, \*\*P<0.001 compared to control samples for each tumour; all other data are not significantly different from control.

3 showed a smaller response and tumour 6 failed to respond at all to either agent. The tumour-promoting phorbol ester TPA stimulated hGH release in four of the six cases, while the non-tumour-promoting phorbol ester PDD had no significant effect in one experiment (tumour 3): hGH release with 100nM PDD was  $121 \pm 6\%$  of control ( $n=4$  replicates,  $P > 0.05$ ), compared to  $193 \pm 25\%$  of control with 100nM TPA ( $n=4$ ,  $P < 0.02$ ). In contrast, hGH mRNA levels showed no consistent changes, with non-significant stimulation by TPA seen only in tumours 2 and 6 (Table 4.2).

#### 4.4 DISCUSSION

In this Chapter the technique of cytoplasmic dot hybridization analysis used in the studies of the single prolactinoma (Chapter 3) has now been applied to study regulation of endogenous hGH mRNA in a series of six GH-secreting tumours. One of the tumours was sufficiently large to yield adequate tissue for RNA extraction for Northern blot analysis. This was an important control experiment, as it demonstrated that the cDNA probe used in the dot hybridization experiments was recognizing a single RNA species of appropriate size in the cultured tumour cells.

The present results show first, that responses of both hGH release and mRNA accumulation are very variable between different tumours but differ from those of normal rat pituitary tissue *in vitro*. As in the case of the prolactinoma studied in Chapter 3, the data show that mRNA levels do not necessarily parallel changes in hormone secretion, and suppression of secretion by somatostatin does not imply suppression of mRNA accumulation.

Growth hormone release and mRNA levels were stimulated in three out of four tumours by GRF 1-29 amide. Previous reports have indicated stimulation of hGH release by equipotent doses of GRF 1-40 and GRF 1-44 in

five out of six tumour cultures, although the increases varied between 30 and 500% above control (Adams et al, 1984). GRF is known to stimulate rGH mRNA levels (Barinaga et al, 1983; Gick et al, 1984; Barinaga et al, 1985) and one previous report describes stimulation of hGH mRNA accumulation by GRF, though only to approximately 35% above control values (Yamashita et al, 1986). Relatively small effects of GRF 1-29 amide were also seen in the present studies, though this could be partly explained by the absence in the culture fluid of supplementary glucocorticoid, which is known to augment somatotroph responsiveness to GRF (Simard et al, 1985; Clayton et al, 1986) or to the relatively short (24h) incubation period. One of the four tumours was completely unresponsive in terms of both hGH mRNA levels and secretion.

Somatostatin and bromocriptine in high doses had clear inhibitory effects on hGH release, confirming the data of Lamberts et al (1987), although the effects of bromocriptine differ from those found with rGH secretion, which was unaffected by dopamine agonists in normal rat pituitary tissue (see Chapter 2). Although hGH mRNA levels were suppressed by bromocriptine in one tumour, no consistent pattern was found for mRNA accumulation in the other three, and somatostatin likewise had no consistent effect on mRNA either. The effects of somatostatin on rGH mRNA accumulation in rat pituitary cells are controversial: using identical experimental techniques and design, one study showed that somatostatin treatment for 24h reduced rGH mRNA levels to 35% of control, comparable to the suppression of rGH release (Wood et al, 1987). However another study found no effect of somatostatin on rGH mRNA levels in the GH<sub>3</sub> cell line (Gick & Bancroft, 1987). Barinaga et al (1985) found no effect of somatostatin on rGH transcriptional rate using a run-on assay, however subsequent studies from

the same group have demonstrated a delayed inhibition of steady state mRNA levels in normal rat pituitary cells (W. Vale, personal communication). One previous report, using a quantitative *in situ* hybridization technique to study three human somatotroph tumours, has described the failure of somatostatin to suppress hGH mRNA levels (Levy & Lightman, 1988). The lack of effect of somatostatin in the present series of six tumours cannot be explained by lack of membrane receptors in view of the inhibition of hGH release in four of the six, and the data might therefore suggest abnormal post-receptor signal transduction.

Cyclic AMP is known to be involved in GRF action, and high intracellular levels of cAMP stimulate rGH release and endogenous rGH gene transcription in normal rat pituitary tissue (Barinaga et al, 1985), and also the transcription of a reporter gene from the hGH promoter region in transfected rat pituitary cells (Brent et al, 1988). Forskolin caused significant stimulation of hGH release and mRNA accumulation in only two tumours. These two tumours also showed the most marked responses to GRF 1-29 amide, implying that the coupling between cAMP generation and hGH gene transcription and hormone release was intact in these cases; on the other hand, the lack of response to either GRF or forskolin in the other tumours might suggest uncoupling at some level between receptor and cellular response. Vallar et al (1987) found a sub-group of about 30% of somatotroph tumours characterized by higher hGH secretion rates and high intracellular cAMP levels: in these tumours adenylate cyclase activity was not stimulated by GRF, and this was related to the presence of a mutated  $G_s$  protein (Landis et al, 1989; and see the discussion in Chapter 8.2.3). In the absence of data for cAMP accumulation it is not possible to say whether the present results indicate two differing types of tumours with



distinctive levels of basal adenylate cyclase activity, although there was no correlation between GRF-responsiveness and the absolute level of basal hGH secretion.

In previous studies of rat pituitary tissue, activation of protein kinase C by phorbol esters was found to stimulate rGH release without increasing rGH gene transcription, in contrast to activation of adenylate cyclase which increased both processes (Barinaga et al, 1985; Morita et al, 1987). In the present study, TPA was able to stimulate hGH release up to four-fold basal levels, demonstrating that protein kinase C can mediate hGH release. However TPA had no consistent effects on hGH mRNA levels suggesting that as in the rat, protein kinase C has no effect on hGH gene regulation.

In conclusion, the present data show that the previously recognized heterogeneity in the patterns of hGH release by pituitary tumour cells *in vitro* also applies to hGH mRNA accumulation. However, more importantly, the data imply that there may be divergences between the regulation of hGH secretion and of mRNA production, as in the case of somatostatin, which could result in the rate of gene transcription being relatively autonomous and independent of receptor-mediated inhibitory control. This recalls the pattern seen with the single prolactinoma described in Chapter 3. Uncoupling between cAMP production and hGH production may be a further factor in a group of somatotroph tumours. Several different mechanisms may underlie the development of adenomatous change in the pituitary gland, including hypomethylation of the hGH gene itself (U et al, 1988), amplified proto-oncogene expression (U et al, 1988) and abnormal signal transduction (Vallar et al, 1987; Landis et al, 1989). The apparent relative autonomy of hGH gene transcription seen in the present study may be related to these

findings and could be involved in tumour pathogenesis. The mechanism for this may be related to amplified proto-oncogene expression, but any further analysis of such factors requires *in vitro* models to study the mechanisms of gene transcription in detail, both in normal and tumourous pituitary cells. The next two chapters will describe the application of transfection analysis and gel retardation analysis to study intracellular regulation of PRL gene expression in rat pituitary cells; Chapter 7 will then return to the topic of pituitary tumours with a presentation of possible applications of these techniques to human adenoma tissue.

## CHAPTER 5

### CALMODULIN-DEPENDENCE OF PROLACTIN GENE EXPRESSION: HYBRIDIZATION AND TRANSFECTION STUDIES

#### 5.1 INTRODUCTION

The hybridization studies described in the earlier chapters provide descriptive information regarding mRNA accumulation, which may be important in understanding how the regulation of hormonal synthesis may differ from that of acute release. The next stage, however, is to address the possible mechanisms for hormonal or intracellular regulation of gene transcription. One well documented example of PRL gene regulation which may be amenable to more detailed study is the role of calcium and calmodulin. In this Chapter the effect of calmodulin antagonists on PRL mRNA accumulation will be described, and then the technique of transient transfection analysis will be used to investigate the role of 5'-flanking sequences from the rPRL gene.

PRL gene expression has a critical requirement for calcium: previous studies have shown that PRL mRNA accumulation (much more than GH mRNA) is highly dependent upon calcium, and both intracellular calcium stores and influx of extracellular calcium are involved (White et al, 1981; Glick & Bancroft, 1985; Hinkle et al, 1988; Davis et al, 1988; Laverriere et al, 1988). Recently, Jackson & Bancroft (1988) have used the technique of transient transfection analysis to show that the proximal 172bp of 5'-flanking DNA from the rPRL gene are able to confer calcium-responsiveness onto a reporter gene, and therefore any calcium-responsive DNA element may

be contained within this part of the proximal enhancer region of the gene.

The effects of calcium on the rPRL gene may be mediated either by protein kinase C or by calmodulin (CaM). Earlier work based on the use of phenothiazine CaM antagonists has suggested that CaM is involved also in regulating PRL mRNA accumulation and gene transcriptional rate (Murdoch et al, 1985a; White, 1985), but these drugs are recognised to have effects on other intracellular signalling systems including protein kinase C itself.

In this Chapter the question of the role of CaM in PRL gene regulation will be addressed using results from two different experimental approaches. First, hybridization studies were used to study the effects of CaM antagonists on mRNA levels as a measure of the control of the endogenous rPRL gene; secondly transient transfection assays were applied to assess the control of transfected rPRL gene promoter sequences by assay of the expression of the chloramphenicol acetyl transferase (CAT) reporter gene. Two specific CaM antagonist agents were employed in the present studies: the highly specific naphthalene sulphonamide CaM antagonist W7 (N-(6-aminohexyl)-1-naphthalene sulphonamide;  $IC_{50}$  approximately 30  $\mu$ mol/L) which has only very slight effects on protein kinase C, and a more potent and more specific iodo-derivative of W7, 5-iodo-1-C8 (abbreviated "W8" here,  $IC_{50}$  approximately 3  $\mu$ mol/L) which has no detectable effect on protein kinase C (MacNeil et al, 1988).

## 5.2 MATERIALS AND METHODS

### 5.2.1 GH<sub>3</sub> cell culture and radioimmunoassays

GH<sub>3</sub> cells obtained from the European Collection of Animal and Cell Cultures (ECACC), were grown in continuous monolayer culture as described in Appendix A1.2. Cells were maintained in Ham's F-10 medium supplemented

with 5% fetal calf serum (Gibco, Paisley, Strathclyde, UK) and 10% horse serum (Gibco). Cells were subcultured from single donor cultures two days before experiments, and then washed with serum-free medium before treatment with test substances in serum-free F-10 medium. Prolactin secretion into culture fluid was measured by radioimmunoassay (as described in Appendix A4.1) using reagents supplied by the National Hormone and Pituitary Program, NIADDK, Bethesda, MD, USA; results are expressed in terms of the standard rPRL-RP3.

#### 5.2.2 Hybridization studies: mRNA measurements

Relative cytoplasmic concentrations of PRL and GH mRNA were measured by cytoplasmic dot hybridization analysis as described in Appendix A3.2. Briefly, plasmid probes containing cDNA inserts specific for rPRL (Gubbins et al, 1979) or rGH (Harpold et al, 1978) were labelled with  $^{32}\text{P}$ -dCTP by nick translation (see Appendix A2.1) to a specific activity of  $1-2 \times 10^6 \text{dpm}/\mu\text{g}$  DNA. Cytoplasmic samples were applied in duplicate to hybridization transfer membranes, usually at 1:10 - 1:50 dilution, membranes were prehybridized and then hybridized with the labelled cDNA probes. After hybridization and autoradiography, mRNA data were quantitated by scanning densitometry and data expressed in arbitrary units of optical density (O.D.) of the developed autoradiographs.

#### 5.2.3 Transient transfection studies

$\text{GH}_3$  cells were transfected with synthetic DNA constructs consisting of 395bp or 958bp of the rat PRL promoter (Lufkin & Bancroft, 1987), or as a control the Rous sarcoma virus (RSV) promoter, linked to the bacterial chloramphenicol acetyl transferase (CAT) gene. Two days before transfection,  $\text{GH}_3$  cells were subcultured onto 100mm Petri dishes at  $4 \times 10^6$  cells per dish. On the day of transfection cells were washed with serum-

free medium, before the addition to each dish of 10µg plasmid DNA in 5ml medium containing 50mM Tris (pH 7.5) and 100µg/ml DEAE dextran. After 30-60 minutes this medium was removed, and the cells washed before addition of fresh medium or test substances for 48h (or for the last 16-24 hours of this period for experiments involving cobalt chloride or calmodulin antagonists).

At the end of the incubation period, medium was removed from the culture dishes, and the cells were washed in ice-cold phosphate buffered saline (PBS). Cells were then harvested by trypsinization (2-3ml of 0.5% trypsin per plate), washed, and lysed with 0.5% Triton X-100 in 0.25mM Tris (pH 7.8). Cell lysates were centrifuged at 15000g for 4 min, and supernatants from the cell lysate were assayed for protein content using the Lowry technique (see Appendix 5.1), and stored for assay of CAT activity.

#### 5.2.4 Assay for CAT activity

CAT activity was assayed as described by Gorman et al (1982) in a 100µl reaction volume containing 100µg cell lysate protein, 12mM acetyl Coenzyme A, and 0.2µCi <sup>14</sup>C-labelled chloramphenicol. Reactions were incubated for 16h at 37°C, and samples were then extracted with an equal volume of ethyl acetate. Acetylation products were separated by thin layer chromatography; samples were spotted onto the TLC plate, which was placed in a sealed tank containing 200ml chloroform:methanol (19:1), and the solvent front allowed to migrate two thirds of the distance of the plate. The plates were air-dried and autoradiographed for 3-4 days. The developed autoradiographs were aligned with the TLC plates to locate the non-acetylated chloramphenicol and its acetylated products; the corresponding spots were cut out from the TLC plates and their radioactivity quantitated by liquid scintill-

ation counting. Results are expressed as percent acetylation of total chloramphenicol/100µg protein.

#### 5.2.5 Materials

W7 (Sigma, Poole, Dorset, UK) and 5-iodo-1-C8 [termed "W8" here (MacNeill et al, 1988)] were initially dissolved in dimethylsulphoxide and freshly diluted in F-10 culture fluid before experiments. Verapamil was purchased from Abbott Laboratories Ltd, Queensborough, Kent, and Bay-K-8644 was a gift from Bayer Pharmaceuticals Ltd. Radiolabelled chloramphenicol was purchased from Amersham International PLC, Amersham, Bucks, UK, and DEAE-dextran from Sigma.

### 5.3 RESULTS

#### 5.3.1 Hybridization experiments

The CaM antagonist W7 caused dose-dependent reduction in PRL secretion, and PRL mRNA levels fell to 26% of control after 24h incubation with 100µM W7 (Figure 5.1). Thyrotrophin-releasing hormone (TRH, 100nmol/L) stimulated both PRL secretion and mRNA levels about two-fold, while W7 (100µmol/L) reduced both PRL secretion and mRNA levels and prevented any stimulation by 100nM TRH (Figure 5.2). GH mRNA levels in these experiments showed slight reductions (to 77-92% of control) in the presence of 100µM W7, but these were much less marked than for PRL mRNA (Figure 5.3). High doses of W7 (100µM and above) caused rounding of GH<sub>3</sub> cells and reduced adherence to the culture dishes, but did not affect cell viability as determined by trypan blue exclusion.

#### 5.3.2 Transfection experiments

GH<sub>3</sub> cells showed no endogenous CAT activity, while cells transfected

with the RSV-CAT construct had high levels of activity, with up to 90% acetylation of chloramphenicol per 100µg protein. Transfection efficiency differed between different experiments, but in general transfection with the PRL-CAT constructs resulted in acetylation of approximately 15% of that seen with the RSV-CAT construct.

In initial experiments to validate calcium responses of the transfected constructs, GH<sub>3</sub> cells were transfected with rPRL(-395)-CAT: treatment with both 50µM verapamil and 0.3mM cobalt chloride dramatically reduced CAT activity to approximately 5% of control levels; on the other hand, treatment with the calcium channel agonist BAY-K-8644 (200nM) modestly increased CAT activity to 148% of control, as has been reported previously (Jackson & Bancroft, 1988; Figure 5.4).

The two CaM antagonists W7 and W8 both markedly reduced CAT activity in GH<sub>3</sub> cells transfected with rPRL(-395)-CAT (Figure 5.5). W8 at 3µM reduced CAT activity to 73% of control levels (mean of two similar experiments), and to  $14 \pm 6\%$  of control at 10µM concentration (mean  $\pm$  S.E.M. from four separate experiments). W7 reduced CAT activity to 6% and 5% of control at 10 and 50µM concentrations respectively (mean of two similar experiments). The degree of inhibition of CAT activity by W8 was similar using both PRL-CAT constructs: using the rPRL(-395)-CAT construct activity was reduced by 10µM W8 to 14% of control levels, and with the rPRL(-958)-CAT construct to 22% of control (means of four and two independent experiments respectively; see Table 5.1 for full data). The effects of CaM antagonists were partially promoter specific: the CaM antagonist W8 (10µM concentration) reduced CAT expression from the RSV promoter to 25% of control, but the inhibition was much greater in the same experiment with the two PRL promoters used (10% of control with rPRL(-958)-CAT, and 7% of control with



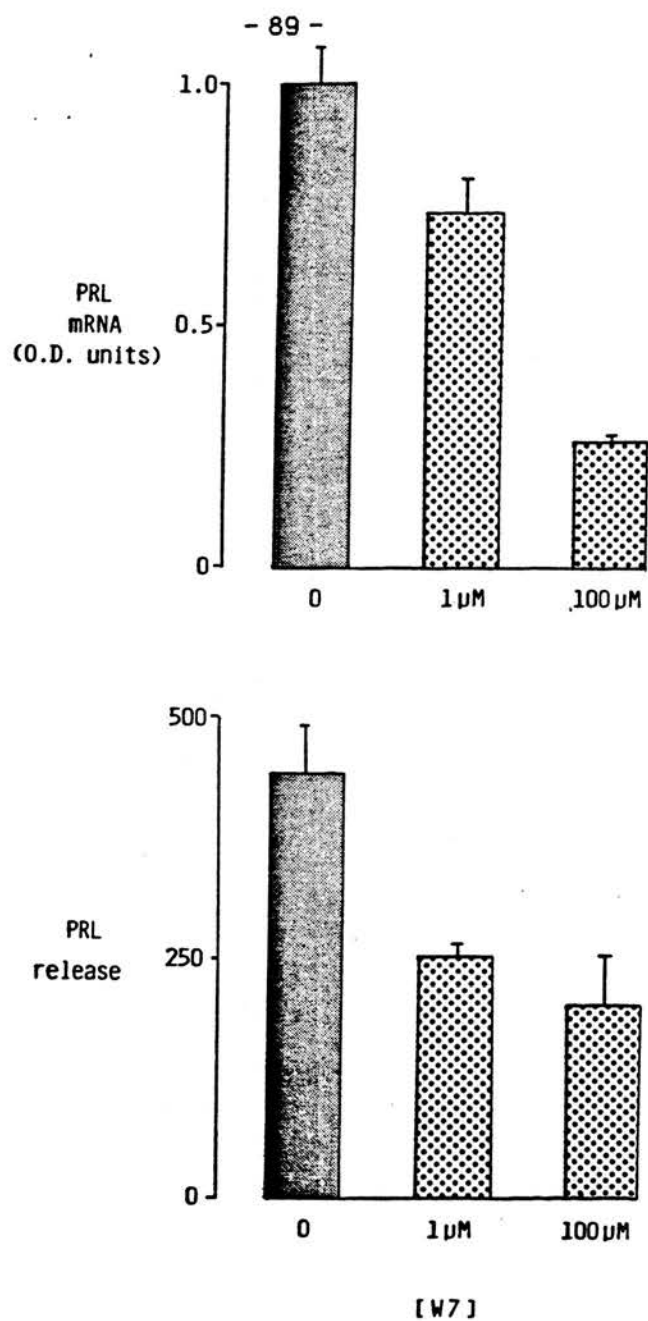


Figure 5.1

Effects of calmodulin antagonist W7 on GH<sub>3</sub> cells. Effects of treatment with 1 or 100 μM W7 for 24h on PRL mRNA accumulation and PRL secretion, n=6 from a single experiment.

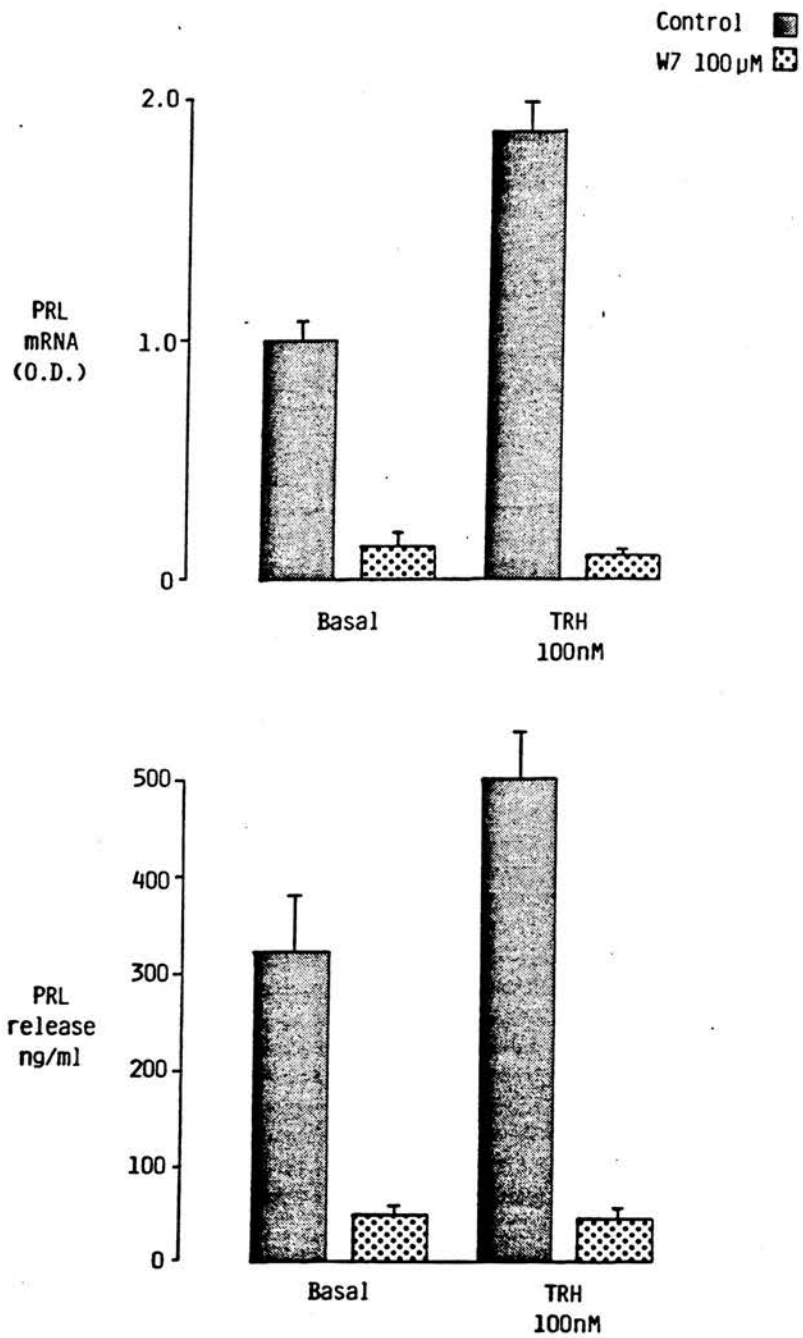


Figure 5.2

Effects of co-incubation with 100μM W7 and 100nM TRH for 24h on PRL mRNA accumulation (n=12 replicates per group pooled from 3 similar experiments,  $P < 0.01$  compared with control in each case).

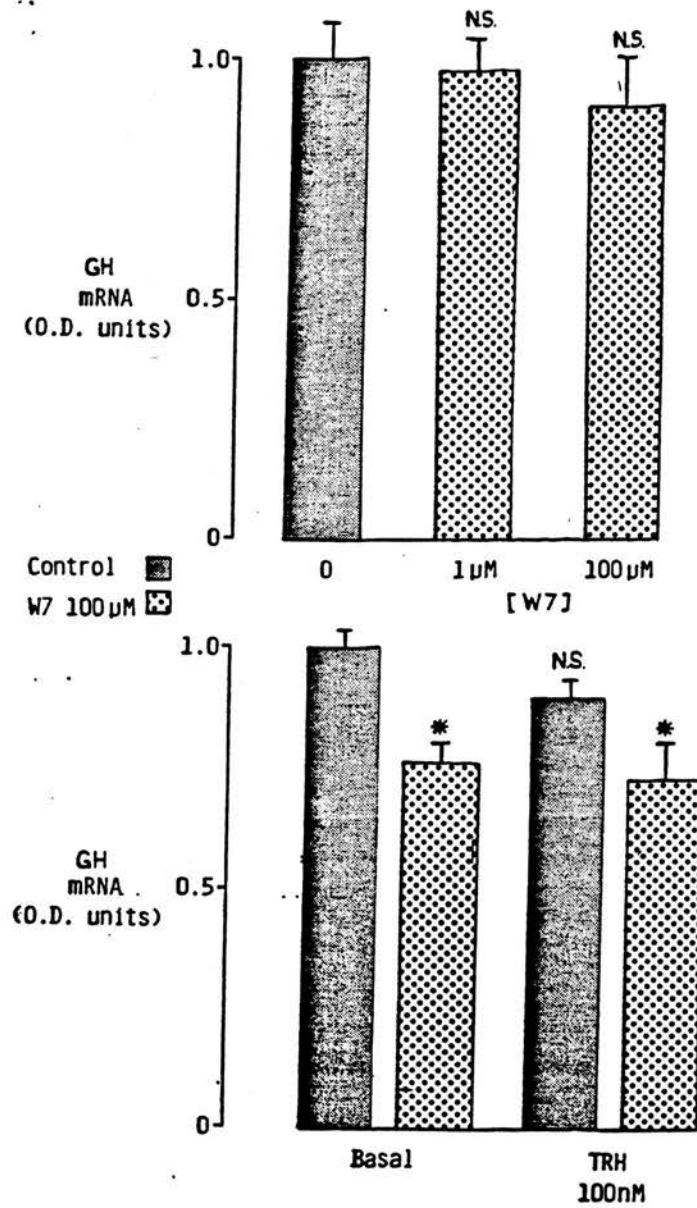


Figure 5.3

Effects of W7 and TRH on GH mRNA in the same experiments; n=6 from the experiment shown in panel A, and n=12 from the experiments shown in panel B, \* P < 0.03 compared to respective controls.

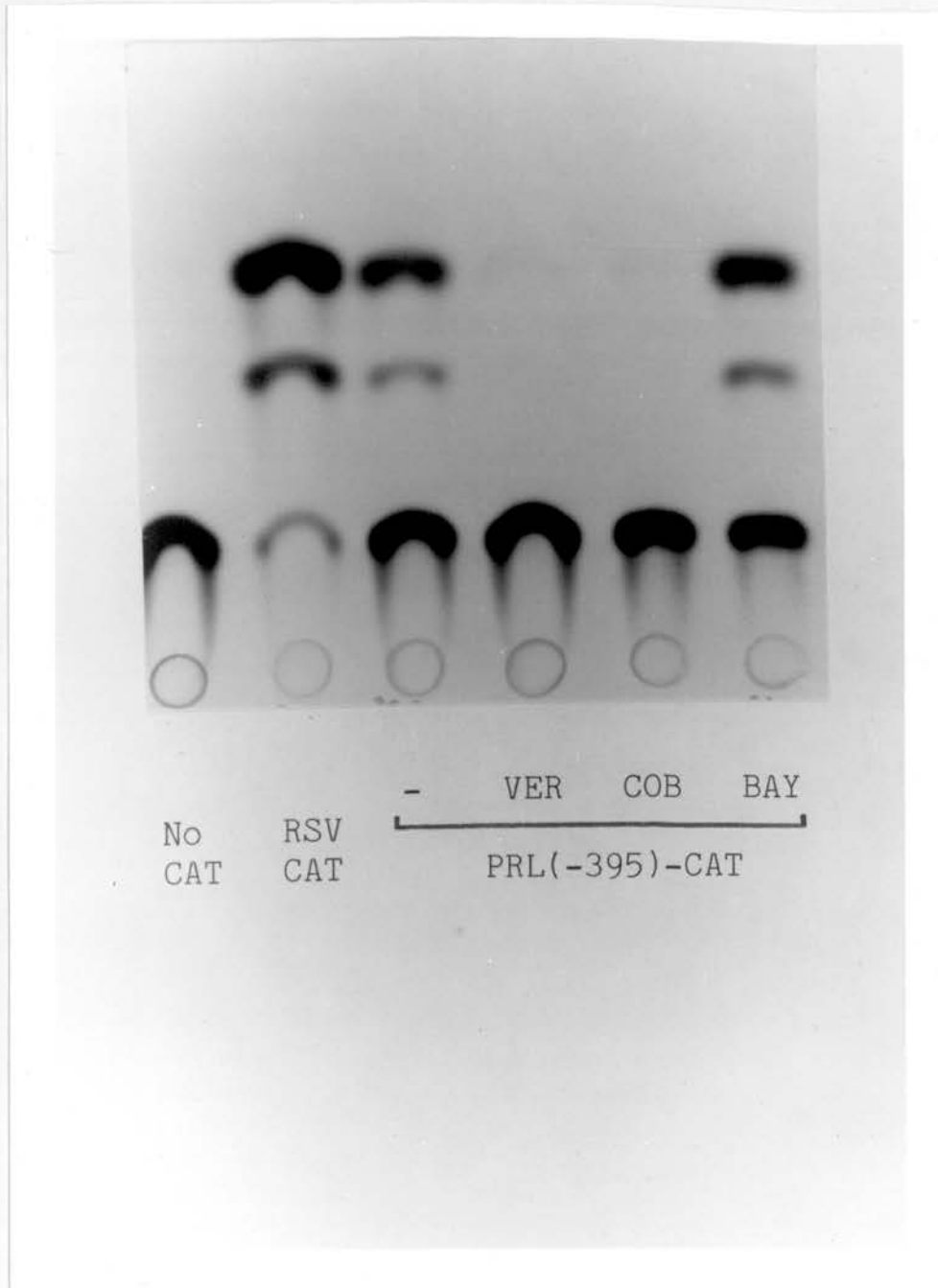


Figure 5.4

Transient transfection of GH<sub>3</sub> cells with RSV-CAT or rPRL(-395)-CAT constructs. Effects of cobalt chloride (0.3mM), verapamil (50μM) or Bay K 8644 (200nM) on CAT expression. The Figure shows results of TLC separation of <sup>14</sup>C-labelled chloramphenicol from acetylated products as a measure of CAT activity in the GH<sub>3</sub> cell extracts.



Figure 5.5

Effects of two calmodulin antagonists (W7 and W8) on acetylation of  $^{14}$ C-chloramphenicol by cytoplasmic extracts of GH<sub>3</sub> cells transfected with rPRL-(-395)-CAT. The first lane shows cells transfected with RSV-CAT plasmid. A single experiment is shown (see text and Table 5.1), and cells were treated with CaM antagonists for 24h before assay for CAT activity.



Figure 5.6

Effects of 10 $\mu$ M W8 on acetylation of  $^{14}$ C-chloramphenicol by cytoplasmic extracts of GH<sub>3</sub> cells transfected either with the RSV-CAT construct, with rPRL(-958)-CAT, or rPRL(-395)-CAT, and treated with W8 for 24h before assay for CAT activity.

Construct	Treatment	Experiment number					
		1	2	3	4	5	6
RSV-CAT	Control	93	-	58	10,4	46,7	-
	W8 10 $\mu$ M					11,5	
rPRL(-395) -CAT	Control	26	1,92	7,8	-	85	33,1
	Verapamil	1,2					
	Bay K 8644	38,5					
	W7 10 $\mu$ M	-	-	0,7	-	-	-
	W7 50 $\mu$ M	-	-	0,7	-	-	-
	W8 3 $\mu$ M	-	-	8,1	-	-	13,8
	W8 10 $\mu$ M	-	0,4	1,9	-	6,1	0,6
rPRL(-958) -CAT	Control	-	-	-	1,1	32,5	-
	W7 10 $\mu$ M	-	-	-	0,15	-	-
	W7 50 $\mu$ M	-	-	-	0,38	-	-
	W8 3 $\mu$ M	-	-	-	0,68	-	-
	W8 10 $\mu$ M	-	-	-	0,37	3,4	-
rPRL(-2400) -CAT	Control	-	-	-	-	8,8	-
	W8 10 $\mu$ M	-	-	-	-	0,7	-

Table 5.1

Transient transfection assays; experimental data for six experiments using calmodulin antagonists, with the % acetylation of  $^{14}$ C-labelled chloramphenicol generated with each construct as determined by thin layer chromatography and liquid scintillation counting.

rPRL(-395)-CAT), implying that the PRL gene promoter is specifically CaM-sensitive and that the proximal 395bp of 5'-flanking DNA accounts for all the CaM responsiveness seen with the longer promoter sequence (Figure 5.6).

#### 5.4 DISCUSSION

In this Chapter, hybridization analysis has been employed to demonstrate the marked calmodulin sensitivity of PRL mRNA accumulation (compared to GH mRNA) in GH<sub>3</sub> cells, and the mechanisms of these effects have been studied using transient transfection assays. The data indicate that highly specific CaM antagonists have major effects which involve 5'-flanking DNA sequences from the proximal enhancer region, within 395 bp of the transcription initiation site.

The precise role of calcium in basal and stimulated PRL gene transcription has been a subject of some controversy. Calcium is an important co-factor for protein kinase C, which is an established stimulator of PRL gene transcription (Murdoch et al, 1985; Elsholtz et al, 1986), but it also activates the calcium-binding protein calmodulin. Recent evidence has shown that down-regulation of protein kinase C by chronic exposure of GH<sub>3</sub> cells to phorbol esters does not affect the increase in PRL mRNA levels induced by calcium (Bandyopadhyay & Bancroft, 1989), suggesting that this kinase is not essential for this effect. Previous studies suggesting that calmodulin (CaM) is important in PRL gene transcription have depended on the use of CaM antagonists such as trifluoperazine and calmidazolium (Glick & Bancroft, 1985; Murdoch et al, 1985; White, 1985) whose specificity is relatively poor as they also inhibit a number of other intracellular enzymes including protein kinase C itself (MacNeill et al, 1988). The hybridization data presented here,



obtained using the naphthalene sulphonamide drug W7, a more highly specific CaM antagonist which has only minimal effects on protein kinase C, confirm that PRL mRNA accumulation is indeed CaM-dependent, in contrast to growth hormone mRNA which was only slightly affected by treatment with W7.

The transfection data showed that the rat PRL gene promoter was efficient at directing expression of the CAT reporter gene in GH<sub>3</sub> cells, although less powerful than the RSV promoter. Chloramphenicol acetylation was readily detected and quantitated, although transfection efficiency varied considerably between experiments. The initial studies confirmed that the two PRL-CAT constructs used were appropriately responsive to calcium channel antagonist and agonist effects, as reported previously by the originator of the constructs (Jackson & Bancroft, 1988), and the more detailed later studies demonstrated that both 395 bp and 958 bp lengths of promoter DNA conferred comparable inhibition on reporter gene expression.

Thus the effects of calcium and CaM appear to be mediated by effects on 5'-flanking DNA in the proximal enhancer region of the rat PRL gene. Response elements have recently been defined for several hormones and intracellular regulators within these enhancer regions (see Chapter 1). For example, the transcriptional response to both EGF and protein kinase C activation by phorbol ester is conferred by DNA sequences between positions -35 and -78 base pairs upstream from the cap site (Elsholtz et al, 1986). A precise response element for calcium itself has not yet been identified, but the information required for a full transcriptional response to calcium is contained within the first 174bp of DNA upstream from the cap site (Jackson & Bancroft, 1988), and our results with Bay-K-8644 and verapamil using the PRL(-395)-CAT construct confirm these findings.

Pharmacological manipulations of the calcium/CaM signalling system are

likely to have wide-spread effects on cellular metabolism, and indeed our data showed that the transcription of CAT from a viral promoter sequence was also substantially reduced by W8. Likewise, three control promoter-CAT constructs tested by Jackson & Bancroft (1988) in fact showed some degree of calcium-induction. However these effects were much different in magnitude compared to the effects on the PRL gene, and in the present study W8 caused more inhibition of CAT expression using the two PRL constructs tested than with RSV-CAT. The proximal enhancer region of the PRL gene therefore appears to contain specific CaM response element(s) which are important in basal and TRH-stimulated gene transcriptional control. However it is important to note that potential response elements may in fact be multiple, and TRH, EGF and cyclic AMP effects have recently been shown to be conferred onto a reporter gene by distal as well as by proximal enhancer regions of the PRL gene (Day & Maurer, 1989b).

The 174bp calcium-responsive stretch of the PRL 5'-flanking DNA contains three of the four proximal binding sites for the pituitary-specific protein factor "Pit-1", (also termed "PUF-1" or "LSF-1") (Gutierrez-Hartmann et al, 1987; Cao et al, 1988; Nelson et al, 1988; Lufkin et al, 1989). The first such site coincides with a response region for EGF and phorbol ester (Elsholtz et al, 1986) and also with a possible recognition sequence for the CaM-dependent enzyme DNA topoisomerase (White & Preston, 1988), inviting the speculation that these intracellular signals may exert their effect by modifying the binding of tissue-specific proteins to DNA or to other DNA-binding factors, or alternatively by modifying already-bound *trans*-acting factors to affect their interaction with the transcription initiation complex. So far there are few data available that address this question. One technique which has been developed to study DNA-protein

interactions is that of the gel retardation assay, and in the next Chapter, this technique will be described and applied to the question of the effects of the CaM signalling system on the PRL gene.

## CHAPTER 6

### STUDIES OF DNA-PROTEIN INTERACTIONS WITH THE PROLACTIN GENE PROMOTER REGION: GEL RETARDATION ASSAYS

#### 6.1 INTRODUCTION

In the last Chapter, the transient transfection assay was used to confirm that a defined region of PRL gene promoter DNA was capable of conferring responsiveness to calcium and calmodulin onto a reporter gene. Further definition of the precise response element would require more detailed studies using deletant constructs containing progressively shorter PRL 5'-flanking DNA sequences, but previous workers have reported difficulty in obtaining adequate levels of basal reporter gene expression in this particular system with shorter deletants (Jackson & Bancroft, 1988). Alternative techniques were therefore sought to assess the functional role of putative response elements, and in this Chapter the potential role of DNA-binding nuclear proteins in calcium/calmodulin control will be addressed using the gel retardation assay; the possible applications of this technique to the study of pituitary tumours will be considered in the next Chapter.

Control of gene transcription is now recognised to involve specific interactions between a number of nuclear proteins and short recognition sequences in the enhancer regions of target genes. Some examples of these interactions are now well characterized, for example the binding of nuclear receptors for thyroid and steroid hormones to their respective response elements to increase the rate of gene transcription. The nature of thyroid

and steroid hormone receptors has been the subject of intense research activity especially since the recognition that they share structural features and certain functional characteristics (reviewed in detail by Evans, 1988). For the rPRL gene, an oestrogen response element (ERE) has been defined in the distal enhancer region, approximately 1500 bp upstream from the transcriptional start site, and immediately adjacent to the first distal site for Pit-1 binding (Maurer & Notides, 1987; Waterman et al, 1988; Kim et al, 1988).

Nuclear proteins are now known to mediate the effects of certain intracellular signalling pathways on gene transcription, and a number of these proteins have recently been studied. Short cyclic AMP response elements (CREs) have been described for several different endocrine genes (for example Tsukada et al, 1987; Montminy & Bilezikjian, 1987; Akerblom et al, 1988), and for one of these cases a CRE-binding protein, CREB, has been purified and cloned (Montminy & Bilezikjian, 1987). A 7 bp phorbol ester (TPA) response element (TRE) has been described which differs from one consensus CRE by only a single base-pair deletion. This TRE binds a protein known as Activator Protein 1 (AP-1), which is encoded by the cellular proto-oncogene *c-jun* (Lee et al, 1987; Curran & Franza, 1988).

Intracellular signalling systems are known to interact at a variety of levels, but additional potential interactions at the level of target genes now seem likely. Binding of nuclear proteins to such response elements may involve protein-protein interactions such as dimerization: AP-1 is known to interact with the proto-oncogene *c-fos*, and the proteins share sequences ("leucine zippers") which would permit dimerization (Karin, 1989). Other protein-protein interactions have been postulated, including AP-1/CREB, AP-1/AP-1, and CREB/CREB, in the regulation of genes such as the pituitary

glycoprotein  $\alpha$ -subunit (Hoeffler et al, 1989), although direct evidence for this is not yet available (Mitchell & Tjian, 1989).

No such nuclear proteins have yet been identified which respond specifically to the calcium/calmodulin signalling system, and indeed no specific calcium response element has yet been identified for the rPRL gene. However the evidence discussed in the last Chapter suggests that any putative calcium/calmodulin response element may reside within the proximal enhancer region of the gene, which contains three Pit-1 binding sites. It was therefore conceivable that these sites might themselves act as response elements for the calcium/calmodulin system, as well as for phorbol ester and EGF, as has previously been shown for the first Pit-1 site (Elsholtz et al, 1986).

This speculation led to the application of a simple assay to study the binding of nuclear proteins to the rPRL proximal enhancer region. The gel retardation assay has been widely established since its first descriptions (Fried & Crothers, 1981; Fried & Crothers, 1984) as a simple and sensitive technique for detection of protein binding to DNA sequences. The technique relies on the fact that complexes of DNA with bound protein migrate through polyacrylamide gels more slowly than unbound DNA fragments. An important advantage of the assay is its greater sensitivity and speed than other techniques such as DNase I footprinting. In addition, the assay provides some information on the number and type of proteins bound, as each distinct complex has a characteristic mobility and specificity, which allows putative interactions between proteins to be studied. The DNA sequence of interest is radiolabelled and incubated with protein extract before separation of complexes by polyacrylamide gel electrophoresis; the

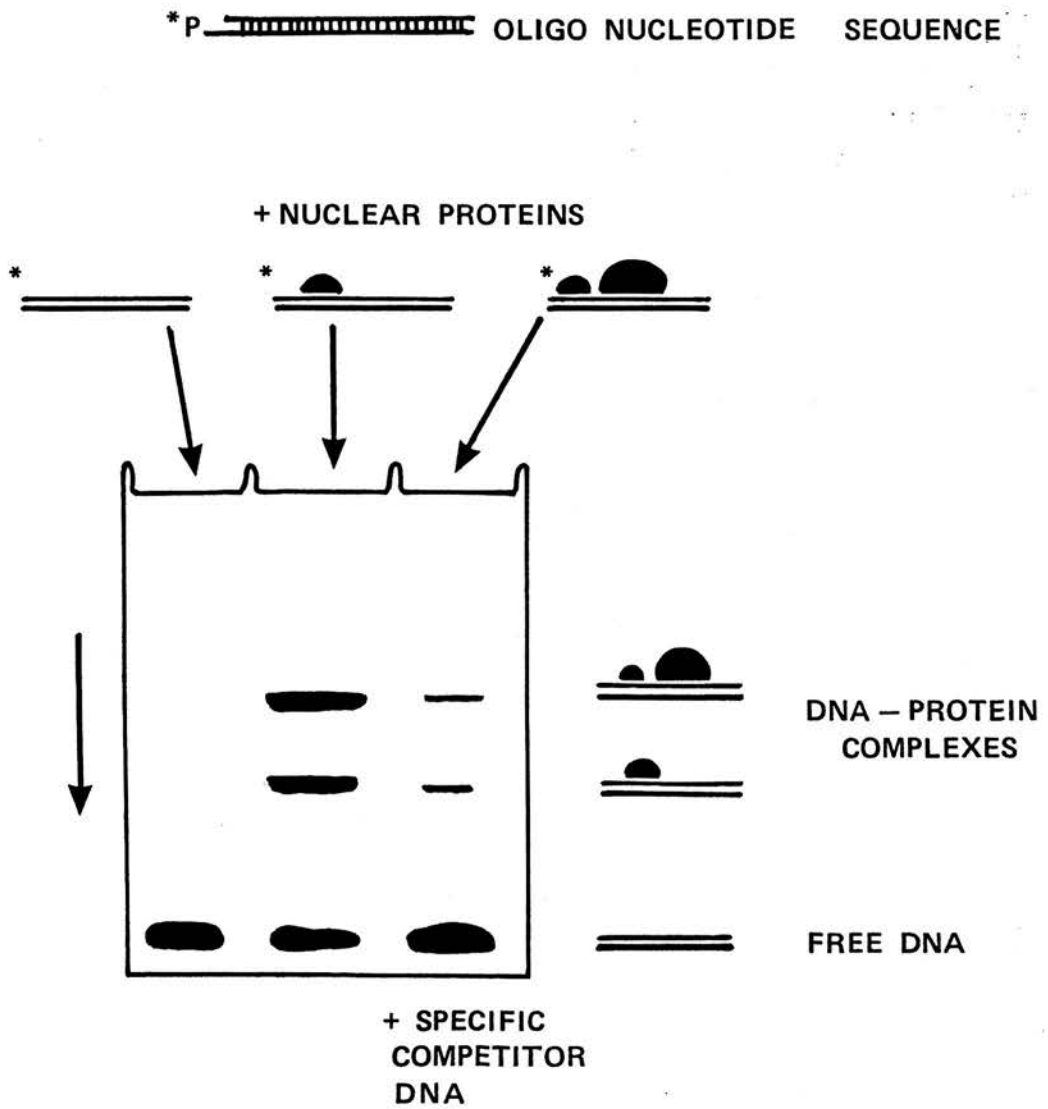


Figure 6.1

Diagram to illustrate the principle of the gel retardation assay.

specificity of the interaction may be tested by competition studies using an excess of homologous or unrelated DNA (see Figure 6.1).

In this Chapter I shall describe the assay, and then present results of preliminary studies of the effects of the calcium/calmodulin signalling system on DNA-protein interactions in GH<sub>3</sub> cells. In the next Chapter, I shall outline some very early attempts to apply this technique to the study of human pituitary tumours.

## 6.2 METHODS

### 6.2.1 Nuclear protein preparation from GH<sub>3</sub> cells

Nuclear proteins were prepared from GH<sub>3</sub> cells, essentially following the method of Ohlsson & Edlund (1986). GH<sub>3</sub> cells were grown to near confluence in 100mm plastic culture dishes, in F-10 culture medium containing 10% horse serum and 5% fetal calf serum.  $1-5 \times 10^6$  cells were first washed in Hanks Buffered salt solution, before being harvested by trypsinization (0.25% trypsin) from the dishes (usually up to 30 dishes of semi-confluent cells). Cells were washed in phosphate-buffered saline, centrifuged at 2300rpm for 10 minutes, and 1 volume of cells resuspended in 4 volumes of buffer containing 10mM Hepes, 15mM KCl, 2mM MgCl<sub>2</sub>, 0.1mM EDTA, 1mM dithiothreitol (DTT) and 1mM phenylmethylsulphonylfluoride (PMSF). Cells were pelleted by centrifugation at 6000 rpm (Beckman SW 50 rotor) and resuspended in 1 ml of buffer. The cells were broken by ten strokes in a hand-held glass-glass homogeniser and the nuclei were pelleted by centrifugation at 10000rpm for 10min at 4°C (Beckman SW 50 rotor). Nuclei were then resuspended in approximately 3ml buffer, and 4M ammonium sulphate solution was gradually added (with constant vortexing) to a final concentration of 0.3M, followed by continued agitation, on ice, for 30 minutes.



The nuclear suspension was next centrifuged at 27,500 rpm for 60 minutes, and the supernatant precipitated by gradual addition of 0.2g ammonium sulphate per ml of supernatant, with constant vortexing and shaking at 4° C. The 0.2g/ml nuclear protein fraction was resuspended in buffer containing 50mM Hepes, 50mM KCl, 0.1mM EDTA, 1mM DTT, 1mM PMSF and 10% (v/v) glycerol, and finally dialysed against 100 volumes of this buffer (with three changes of buffer at 4°C over 4 hours). The dialysed extracts were stored at -70°C or in liquid nitrogen for use in the gel retardation assay.

For the studies of the effects of calmodulin antagonists, nuclear protein extracts were prepared from GH<sub>3</sub> cells which were either grown in serum free culture medium for 24 hrs, or treated for 24 hrs with 50µM W7 in serum-free medium, or for 4 hrs with 10µM W8 before protein extraction.

#### 6.2.2 Gel retardation analysis

Restriction fragments from the 5'-flanking region of the rPRL gene were generated by digestion of the PRL(-395)-CAT construct with Hae III and Ava II (-172/+33 fragment). In addition a synthetic 24bp oligonucleotide sequence was studied (positions -162/-138, denoted "rPRL-P3"), containing the third proximal binding site of the pituitary-specific protein Pit-1. The sequence of this oligonucleotide is:

5' -GATCCCTTCCTGAATATGAATAAGAAATAAAAG-3'  
3' -GGAAGGACTTATACTTATTCTTTATTTTCCTAG-5'

Restriction fragments were end-labelled with  $\alpha^{32}\text{P}$ -dCTP using the Klenow fragment of DNA polymerase, and rPRL-P3 was labelled with  $\gamma^{32}\text{P}$ -ATP using the forward reaction catalysed by T4-polynucleotide kinase (see Appendix A2.3).

For the gel retardation assay approximately 1ng of labelled rPRL-P3 was incubated with 10µg nuclear protein extracts in the presence of 3µg poly-(dI.dC) as a synthetic non-specific competitor DNA, with or without unlabelled excess oligonucleotide or restriction fragments in specific competition studies as indicated. Binding reactions were allowed to proceed for 15-30 minutes at room temperature in a total volume of 20µl in a buffer containing 150mM KCl, 25mM Hepes, 0.1mM EDTA, 1mM DTT 1mM and 10% glycerol. DNA-protein complexes were then separated by electrophoresis on a 7% polyacrylamide gel in 25mM Tris-188mM glycine buffer before autoradiography for 1-8 hours.

### 6.3 RESULTS

#### 6.3.1 Effect of GH<sub>3</sub> nuclear protein on PRL -172/+33 fragment

In initial experiments, restriction fragments from the proximal enhancer region of the rat PRL gene were prepared and end-labelled with <sup>32</sup>P-dCTP. Incubation of labelled fragment (-172/+33) with GH<sub>3</sub> cell nuclear extracts resulted in formation of DNA-protein complexes which migrated more slowly than free DNA on gel electrophoresis (Figure 6.2). This complex formation was reduced by competition with an excess of rPRL-P3 oligonucleotide or of unlabelled PRL -172/+33 fragment itself, but not by an excess of unrelated oligonucleotide from the human insulin insulin gene promoter (kindly provided by Dr D. S. W. Boam, Birmingham).

However these experiments were somewhat unsatisfactory in that poor resolution of DNA-protein complexes was achieved. The region -172/+33 within the proximal enhancer region, which is reported to contain a calcium responsive element (as described in Chapter 5), contains three binding sites for the pituitary-specific protein, Pit-1 (Nelson et al, 1988). We

therefore chose to perform more detailed gel retardation studies using this oligonucleotide rPRL-P3 (-138/-162) as the labelled DNA sequence; this represents the third such site within the proximal enhancer region, and has high affinity for Pit-1 binding (Nelson et al, 1988).

#### 6.3.2 Effect of GH<sub>3</sub> nuclear protein on PRL-P3 oligonucleotide

Incubation of radiolabelled rPRL-P3 oligonucleotide with GH<sub>3</sub> cell nuclear protein extract resulted in multiple retarded DNA-protein complexes on gel electrophoresis. This protein binding was markedly reduced by competition with a molar excess of unlabelled rPRL-P3 or an excess of restriction fragment -172/+33 from PRL 5'-flanking DNA (Figure 6.3). That this competition was sequence specific was suggested by the lack of competition from even a 300-fold excess of mutated oligonucleotide from the human insulin promoter ("Ins 5'").

#### 6.3.3 Effect of calmodulin antagonists on nuclear protein binding

The effects of the calmodulin antagonists W7 and W8 were next examined. In initial experiments, GH<sub>3</sub> cells were treated for 24 hours with 50μM W7 before protein extracts were prepared; however, because of concerns as to the specificity or possible undetected toxicity of treatment with W7 over long periods, separate nuclear protein extracts were prepared from GH<sub>3</sub> cells treated for just 4 hours with 10μM W8. Both treatment protocols gave similar results, with a dramatic overall reduction in the number of retarded complexes seen, and the appearance of a new low-mobility retarded band which was not visible with control GH<sub>3</sub> extracts and whose formation was competed by excess unlabelled rPRL-P3 (Figure 6.4).

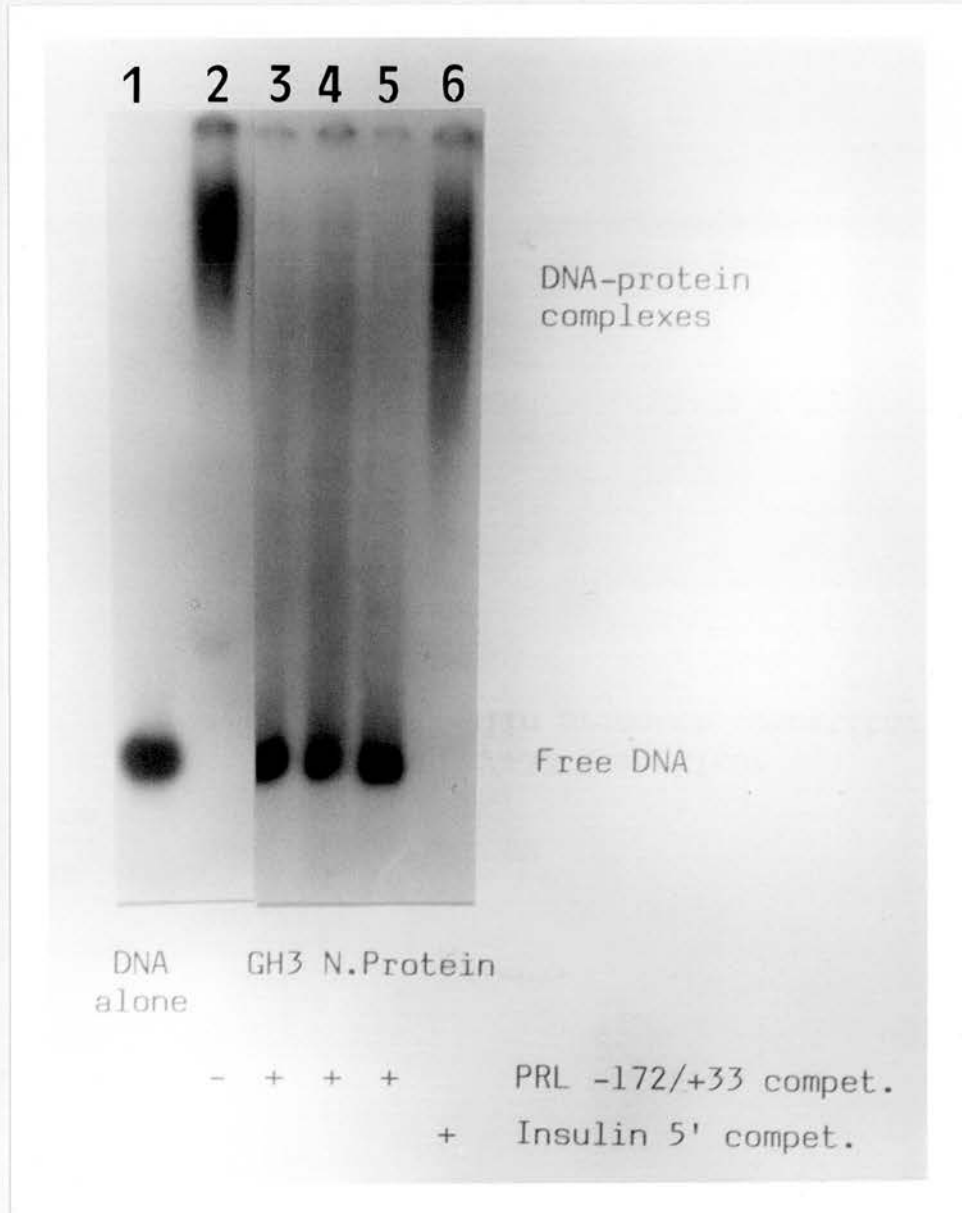


Figure 6.2

Gel retardation patterns seen with  $^{32}\text{P}$ -labelled restriction fragment PRL -172/+33 with GH3 nuclear protein extract. The migration of labelled DNA without any protein extract is shown in lane 1, and with 10 $\mu\text{g}$  GH<sub>3</sub> protein extract in lane 2. Fragment binding was competed by 10- 20- and 100-fold excess of unlabelled PRL -172/+33 fragment itself (lanes 3-5), but not by an excess of unrelated oligonucleotide from the insulin gene promoter (lane 6).

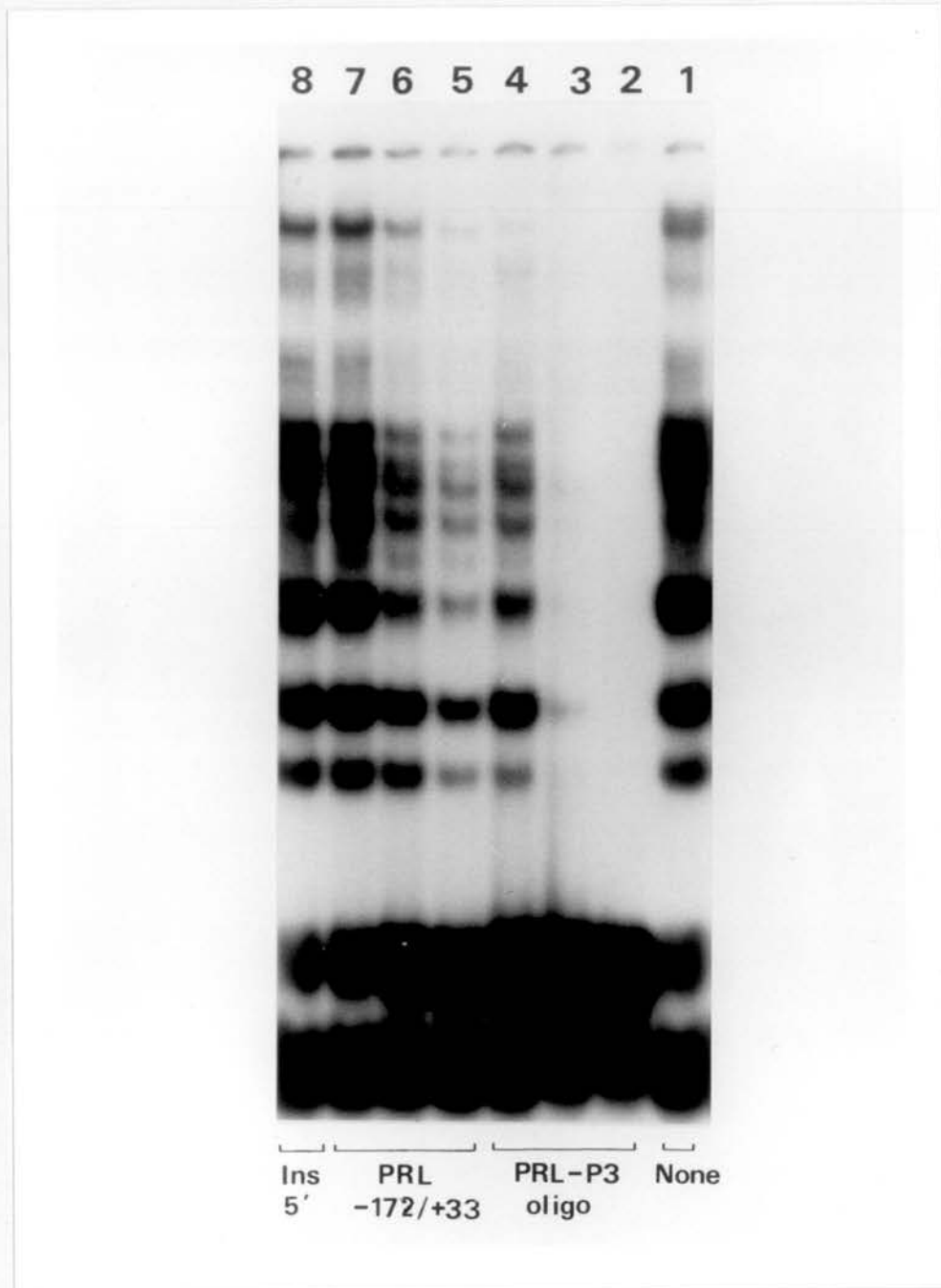


Figure 6.3

Gel retardation patterns seen with  $^{32}$ -P-labelled PRL-P3 oligonucleotide incubated with nuclear protein extracts from GH<sub>3</sub> cells alone (lane 1), or competed with 300-, 30- or 3-fold molar excess of unlabelled PRL-P3 (lanes 2-4) or with excesses of rPRL restriction fragment -172/+33 (lanes 5-7), or with 300-fold excess of human insulin oligonucleotide (Ins-5', lane 8).



Figure 6.4

Effect of treatment of GH<sub>3</sub> cells with calmodulin antagonists on protein-DNA complex formation. Cells were treated with either 50 $\mu$ M W7 for 24 hours, or 10 $\mu$ M W8 for 4 hours before nuclear protein extraction and protein incubation with labelled oligonucleotide PRL-P3. Migration of free DNA is shown in lanes 1, 3 and 5; incubation of PRL-P3 with control protein (untreated GH<sub>3</sub> cell extract, "Con") in lane 2. Proteins derived from cells treated with W7 or W8 are shown in lanes 4 and 6 respectively, and in lane 7, the reaction contained a 50-fold excess of unlabelled PRL-P3 as competitor DNA (indicated +).

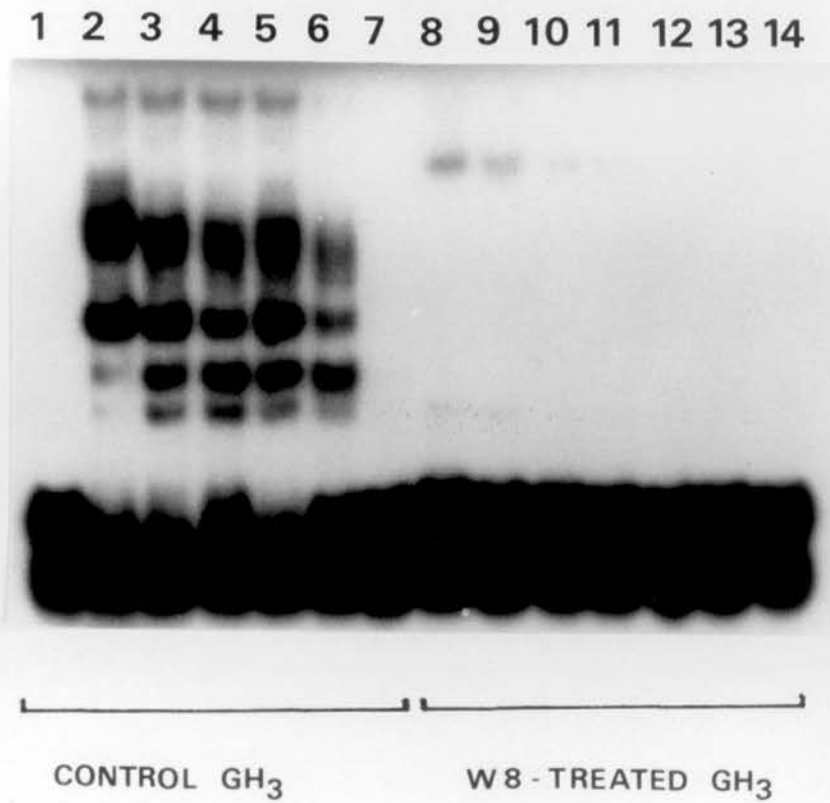


Figure 6.5

Effect of treatment of GH<sub>3</sub> cells with 10µM W8 for 4h before nuclear protein extraction on gel retardation patterns with labelled oligonucleotide PRL-P3. Lanes 1 and 14 show the migration of free labelled DNA without added nuclear protein; protein concentrations from control and W8-treated cells were 10, 5, 2.5, 1µg, 0.5µg and 0.1µg in lanes 2-7 and 8-13 respectively).

The overall apparent reduction in DNA-protein binding induced by W8 treatment was detectable at a variety of different doses of nuclear protein in the DNA-protein binding incubation reaction, but the appearance of the low-mobility complex was only detectable with at least 2.5 $\mu$ g protein, in contrast to the major complexes seen with protein extracts from untreated GH<sub>3</sub> cells which could be detected with as little as 0.5 $\mu$ g protein in the incubation reaction (Figure 6.5).

#### 6.4 DISCUSSION

In this Chapter the technique of gel retardation analysis of DNA-binding proteins has been described, and applied in an attempt to answer the questions raised in Chapter 5 regarding the mechanism of calmodulin action.

Gel retardation analysis is a simple and highly sensitive technique for detecting binding of proteins to target sequences of DNA. The biophysical nature of this *in vitro* protein-DNA interaction is not yet fully understood. For example it appears that proteins do not dissociate from DNA once entrapped in the gel (the "cage effect"), and a small number of DNA binding proteins may in fact give rise to a several different possible complexes, with a relatively large number of different binding states (Cann, 1989). Nonetheless, it is now becoming widely used as a tool for identifying DNA sequence-specific and tissue-specific protein binding activities in nuclear extracts.

The results described here show that GH<sub>3</sub> cell nuclear extracts contain a protein factor that can bind specifically to proximal 5'-flanking sequences from the rPRL gene. Competition studies using homologous and unrelated DNA sequences confirmed DNA-specific binding of the pituitary nuclear protein extracts. (Further studies using extracts from different tissues are



described in the next Chapter.) The most striking finding was that exposure of GH<sub>3</sub> cells to two calmodulin antagonist drugs dramatically altered the pattern observed. The findings are important because they offer direct evidence that the binding efficacy of nuclear proteins may be directly affected by manipulation of an intracellular signalling pathway which is known to mediate the effects of extracellular hormones. Thus an extracellular signal may exert effects on gene transcription by modifying the binding of transcription factors to hormone response elements.

One previous report described a similar experimental protocol, showing that the pattern of binding of pituitary nuclear proteins to the EGF/phorbol ester response element (site 1P) was unaffected by a 1-hour exposure of cells to phorbol ester (Elsholtz et al, 1986). For some transcription factors, their transcriptional activity of DNA-binding proteins may be mediated by allosteric modifications of proteins already bound to DNA (Montminy & Bilezikjian, 1987; Roesler et al, 1988; Mitchell & Tjian, 1989). However studies on CREB have shown that cAMP may enhance DNA binding and transcriptional activity (Yamamoto et al, 1988). A recent report has provided evidence that exposure of cells to insulin induces the appearance of a new protein that binds to the hGH promoter in gel retardation assays; this DNA binding factor was not seen in untreated cells (Prager et al, 1990). Hence it may be that intracellular signals are able to modulate DNA-binding activity of transcription factors as one mechanism of controlling gene transcriptional rate.

The present results show that exposure of pituitary cells to specific calmodulin antagonists dramatically alters the pattern of protein binding to the high affinity site 3P, suggesting that protein binding to DNA may itself be calmodulin-sensitive. The proteins involved are as yet

uncharacterized, but further studies should clarify whether calmodulin itself may be involved in complex formation, and also the effects of the calcium-CaM system on protein binding to the other sites known to bind the pituitary-specific factor Pit-1.

The studies presented in this Chapter have applied a simple technique for the detection of pituitary nuclear protein interactions with DNA sequences of interest. The results presented here were performed to address the questions raised in Chapter 5 about the possible mechanism of action of calmodulin in regulating PRL gene expression. However the technique has a variety of other potential applications. In particular, the question was raised in Chapters 3 and 4 of possible abnormalities of hormone gene regulation in pituitary tumours. If it is the case that, for example, the PRL gene is constitutively activated in certain prolactinomas, it may be that abnormal *trans*-acting factors are responsible. The sensitivity of the gel retardation assay may allow detection of abnormal protein-DNA binding patterns, and preliminary work in this direction will be presented in Chapter 7.

## CHAPTER 7

### APPLICATION OF TRANSIENT TRANSFECTION AND GEL RETARDATION ASSAY TO HUMAN TISSUES: PITUITARY AND PLACENTA

#### 7.1 INTRODUCTION

The techniques of transfection analysis and gel retardation analysis may be useful in studies of gene regulation in human pituitary tumours. Transient transfection assays may be used to compare the regulation of exogenous (i.e. transfected) promoter sequences by hormonal manipulations of cultured cells, which may be either from rat pituitary cell lines or primary cultures of the pituitary tumours themselves. Such a technique might therefore be used to assess to what extent the tumour cells contain the machinery to allow appropriate regulation of a defined promoter sequence. Regulation of the endogenous human growth hormone gene in human somatotroph adenoma cells (by insulin, thyroid hormone and dexamethasone) has been shown to differ from regulation of the promoter region transfected into rat pituitary GC cell lines (Isaacs et al, 1987), implying that some aspect of the intracellular environment was involved in determining the transcriptional response. However there have been no reports so far of transfection of human pituitary tumour cells themselves, and this might provide more relevant information if it is feasible.

In the last Chapter, the technique of gel retardation assay was described in relation to the calcium/calmodulin dependence of PRL gene expression in the GH<sub>3</sub> rat pituitary cell line. As suggested at the end of that Chapter, this technique also may be applicable to the study of human pituitary tumours. If it is hypothesized that expression of pituitary

hormone genes may in some tumours be constitutively activated and not susceptible to normal hormonal suppression, then the gel retardation assay may offer a simple system for assessing the presence or absence of certain proteins capable of binding to hormone gene response elements such as Pit-1 or AP-1. It has the advantage of greater sensitivity than the DNase I footprinting technique, and also requires much less nuclear protein extract.

In this Chapter a preliminary and unsuccessful attempt to carry out transient transfection of a human prolactinoma will be described. Then a technique of nuclear protein extraction from small amounts of human tissues will be described, leading to the successful application of the gel retardation assay using these extracts in preliminary studies of nuclear proteins binding to a hPRL gene promoter sequence. The initial studies used nuclear proteins derived from bovine and post-mortem human pituitary tissue from patients who died of non-endocrine causes, and compared these extracts with that derived from a pituitary tumour. Further studies were also performed to demonstrate the presence of specific DNA-binding proteins in human placental tissue. The nuclear protein extractions and gel retardations described here were all carried out during a four week period as a visiting fellow in the laboratory of Dr Alexandra Belayew and Prof Joseph Martial, Université de Liège, Belgium.

## 7.2 METHODS

### 7.2.1 Transient transfection of human prolactinoma cells

Prolactinoma tissue was obtained from the patient M.H., whose clinical history was summarised in Chapter 3. The data for hPRL mRNA accumulation derived from experiments carried out on tissue obtained at hypophysectomy

in June 1986 (see Chapter 3.2), while the present experiments were done on tissue from the patient's third hypophysectomy operation in 1988.

The tissue was enzymatically dispersed as described in Appendix A1.1, to yield approximately  $6 \times 10^6$  viable cells. These were plated out into multiwell culture dishes at  $5 \times 10^5$  cells per well, in Dulbecco's modified Eagle's medium containing 10% horse serum and 2.5% fetal calf serum with antibiotics. After 7 days in culture the cells were only partially adherent to the culture dishes, but it was decided to attempt a transient transfection using the DEAE-dextran method. Two constructs were employed: the first consisted of the proximal 740 bp from the hPRL gene 5'-flanking sequence linked to the CAT reporter gene, and the second consisted of the proximal 500 bp from the hGH gene promoter linked to the CAT gene (both kindly provided by Dr Alexandra Belayew and Prof Joseph Martial, Laboratoire de Génie Génétique, Université de Liège, Belgium). Because of the small numbers of cells available, RSV-CAT was not used as an internal assessment of transfection efficiency.

The transfection procedure was carried out as described in Chapter 5.2.3, with extreme care taken during the washing stages to avoid loss of poorly adherent cells from the dishes. Approximately  $1.5 \times 10^6$  cells were used in each group, pooled from several culture wells. Two groups of cells were transfected with the hPRL(-740)-CAT construct and were treated after transfection with either control serum-free medium or with medium containing 100nM bromocriptine for 48 hours before cell harvesting for the CAT assay. One group of cells were transfected with hGH(-500)-CAT and were left untreated.

The cells were finally harvested and cytoplasmic extracts prepared for the CAT assay, which was performed as described as in Chapter 5.2.4.

### 7.2.2 Normal bovine pituitary tissue: nuclear protein extraction

Bovine pituitaries were obtained from the Liège abattoir, and were kept on ice after removal for 30 minutes before commencing the nuclear protein extraction procedure.

The procedure for extracting nuclear proteins from cultured cells was not directly applicable to intact tissues, therefore a technique was modified for use with small amounts of tissue from a published report (Gorski et al, 1986) describing the preparation of transcriptionally active protein extracts from various mouse tissues. The buffers used in this technique are viscous to ensure separation of nuclei from other organelles after homogenization, and have low salt concentrations (with EDTA included in initial homogenization buffers to remove magnesium and calcium ions, to preserve chromatin structure) spermine and spermidine are included to replace ionic positive charges.

Tissue was chopped with a scalpel blade, and then homogenized using a 30ml teflon-glass motor-driven homogenizer in 15-30ml homogenization buffer [10mM Hepes, pH 7.6; 25mM KCl; 0.15mM spermine; 0.5mM spermidine; 1mM EDTA; 2M sucrose and 10% glycerol]. The homogenate was layered over a 10ml cushion of homogenization buffer, and the nuclei pelleted by centrifugation at 24,000 r.p.m. for 30 minutes in an SW28 rotor at 0°C. The nuclei were resuspended in fresh buffer and counted at this stage: from 3 bovine pituitary glands (approximately 5 grams of tissue),  $20 \times 10^6$  nuclei were obtained. The resuspension buffer was identical to homogenization buffer apart from containing additional glycerol (made up to 20%) and reduced sucrose (200mM, to allow more complete removal of the viscous supernatant) and the nuclear suspension was recentrifuged for 1-5 minutes at 11,000 r.p.m. in a JA20 rotor (16,000g).

The nuclei were then resuspended in 300 $\mu$ l nuclear lysis buffer [10mM Hepes, pH 7.6; 100mM KCl; 3mM MgCl<sub>2</sub>; 0.1mM EDTA; 1mM dithiothreitol (DTT); 0.1mM phenylmethylsulphonyl fluoride (PMSF), and 10% glycerol - DTT and PMSF were added immediately before use], and manually homogenized using a glass-glass homogenizer. An aliquot of the homogenized material was taken and its absorbance read at A<sub>260</sub>, before the remaining material was extracted using ammonium sulphate to precipitate chromatin. 4M ammonium sulphate (pH 7.9) was slowly added until reaching 10% of the total volume, and the viscous material gently shaken on ice for 30 minutes before centrifugation at 45,000 r.p.m for 2 hours in a Ti50 rotor. The supernatant was finally dialysed overnight against 2 changes of 250ml dialysis buffer [25mM Hepes (pH 7.6); 40mM KCl; 0.1mM EDTA; 1mM DTT, and 10% glycerol].

The concentrations of nuclear proteins after dialysis was 3-4 $\mu$ g/ $\mu$ l, and aliquots of the protein extract were stored in liquid nitrogen before use in gel retardation assays.

#### 7.2.3 Normal human pituitary, pituitary tumour and placenta: nuclear protein extraction

Normal human pituitaries were obtained from post-mortems carried out within three hours of death from patients who had died of non-endocrine disease. After initial experiments, one of these was used for protein extraction exactly as described in section 7.2.2. The patient was a man aged 58 years, who had died of myocardial infarction. The pituitary gland was stored in liquid nitrogen before protein extraction, and weighed 0.6g.  $2 \times 10^6$  nuclei were obtained, and the final protein concentration was approximately 0.25 $\mu$ g/ $\mu$ l.



A single pituitary tumour was subjected to nuclear protein extraction in parallel with the normal pituitary tissue. The tumour was a TSH-secreting macroadenoma from a 49 year old woman (G.McN.) who presented with thyrotoxicosis, with high levels of serum TSH. The tissue was kindly made available for study by Dr W.A. Burr (Consultant Physician) and Mr Towns (Consultant Neurosurgeon), Pinderfields Hospital, Wakefield. The patient had partly responded to somatostatin analogue therapy, but was then treated by a trans-sphenoidal hypophysectomy. Some of the tumour tissue was grown in monolayer cell culture, and this had shown that TSH secretion *in vitro* was unaffected by treatment with T3, but reduced to 50% of control with 1 $\mu$ M somatostatin (data not shown). 3 x 10<sup>6</sup> nuclei were isolated, but the final protein concentration was just detectable at approximately 0.1 $\mu$ g/ $\mu$ l.

Finally, a nuclear protein extract was prepared from a normal human placenta. The material used weighed 23g, and gave a final protein concentration of 0.2 $\mu$ g/ $\mu$ l.

#### 7.2.4 Gel retardation assays

These assays were carried out precisely as described in Chapter 6. The oligonucleotide was the same as that used in those studies of rat pituitary proteins, there termed rPRL-P3; the same sequence is located in a similar position in the human prolactin proximal enhancer region, and is identical to the second of the three pituitary-specific protein binding sites (putative Pit-1/GHF-1 binding sites) described in the proximal enhancer region of the human prolactin gene (Lemaigre et al, 1989). Hence in these studies it is termed hPRL-P2, even though the sequence is identical. For comparison with the tissue protein extracts, previously prepared nuclear protein extracts from GH<sub>3</sub>B6 cells were used; these extracts were prepared



using the technique described by Dignam et al (1983), and so differ slightly from those described in Chapter 6.

Competition studies were performed using either an excess of unlabelled hPRL-P2, or of an unrelated oligonucleotide (the consensus binding site for the CCAAT enhancer protein, C/EBP).

### 7.3 RESULTS

#### 7.3.1 Transient transfection of prolactinoma cells

Cytoplasmic preparations were made from the cultured cells from this tumour for assay of CAT activity. Chloramphenicol was separated from its acetylated products by thin layer chromatography as before, and the autoradiogram is shown in Figure 7.1. The levels of acetylation were very low, and did not vary among the three treatments studied. Cytoplasmic extracts from cells transfected with hPRL(-740)-CAT and treated with control medium generated 3.8% acetylation/mg protein, and those treated with 100nM bromocriptine gave an identical result. Extracts from cells transfected with hGH(-500)-CAT gave 3.3% acetylation/mg protein.

These are very low levels of chloramphenicol acetylation, and suggest that the transfection efficiency or cell viability after transfection was very poor. This may reflect the fact that cells were only poorly adherent, or that the absolute numbers of cells were inadequate to give reliable results. Thus unfortunately the data are not adequate to draw any conclusions as to the ability of the prolactinoma cells to respond to dopaminergic control of transcription of the transfected promoter construct for comparison with the data for mRNA presented in Chapter 3. However, despite the technical problems experienced, it seems likely that transient transfection of adequate primary cultures of pituitary tumour cells may be

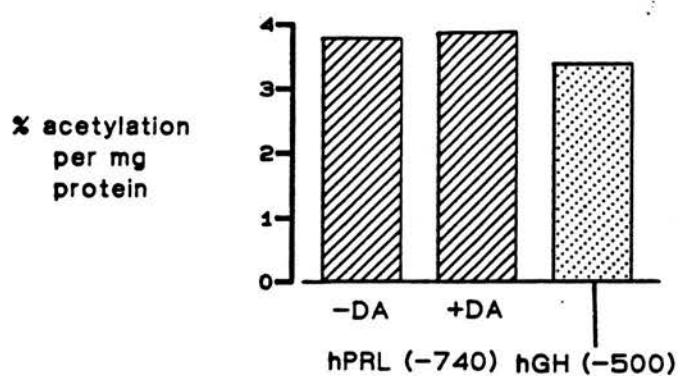


Figure 7.1

Transient transfection of cultured human prolactinoma cells. Cross-hatched bars: hPRL(-740)-CAT, control medium (-DA) and 100nM bromocriptine (+DA); stippled bar: hGH(-500)-CAT, control medium.

possible, and that valuable data may be obtainable about control of gene transcription in these tumours.

### 7.3.2 Gel retardation studies

In initial experiments, bovine pituitary extract was compared to nuclear extracts previously prepared (Bernard Peers, Laboratoire Central de Génie Génétique, Liège, Belgium) from rat pituitary GH<sub>3</sub>B6 cells by the procedure described by Dignam et al (1983). A variety of concentrations of KCl were employed, as the salt concentration is thought to be critical for protein binding to DNA (Ausubel et al, 1989). A number of retarded DNA-protein complexes were seen with both extracts, but the extent of different complex formation was affected by KCl (Figure 7.2). An artefactual (non-competable) low mobility band was often seen at the top of gels, in these and subsequent experiments, sometimes even in the absence of protein, but this complex was not generally seen with KCl concentrations of 75mM or higher, and its significance remains uncertain. At least three faster-running (higher mobility) bands were seen with both extracts, two of them apparently identical with the different extracts, and one which was of higher mobility with bovine pituitary extract than with GH<sub>3</sub>B6 extract, and all of these bands were reproducibly and easily detected with KCl concentrations between 75 and 150mM (Figure 7.2). Thus these experiments indicated that a technique for extracting nuclear protein from fresh tissue could be applied to small quantities of pituitary tissue, and gave results comparable to the technique of Dignam et al (1983) for cultured cells.

In the next experiments, bovine pituitary extract was used for comparison with nuclear protein extracts (0.2-2.0µg) prepared from a normal human pituitary gland obtained post-mortem, and with proteins prepared from human placenta. These experiments were performed with [KCl] of 50mM,

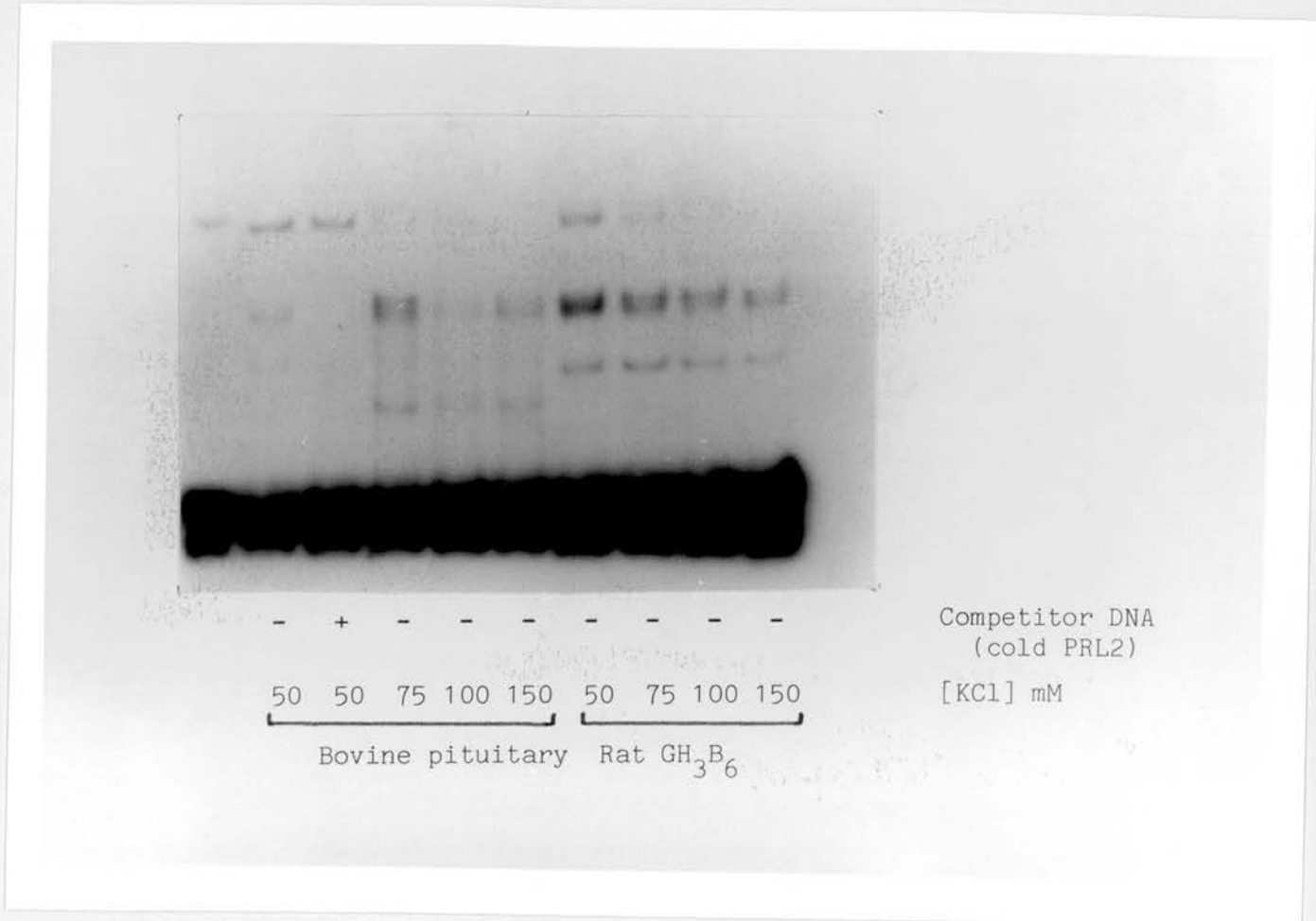


Figure 7.2

Gel retardation analysis, comparing nuclear proteins extracted from bovine pituitary tissue or rat GH3B6 cells. Lane 1, DNA (hPRL-P2) alone; lanes 2-10, DNA incubated with 10µg nuclear protein. In lane 3, a 50-fold molar excess of unlabelled hPRL-P2 was included. KCl concentrations were varied as indicated.

before this parameter was varied, and the previously noted artefactual low-mobility band was a problem with these gels. However, specific bands representing true DNA-protein complexes were seen with all three extracts, and the pattern seen with human pituitary strikingly resembled that found with bovine pituitary extracts with at least three retarded bands of identical electrophoretic mobility (Figure 7.3). The pattern seen with placental extracts differed: at least one of the two "pituitary" bands (band 1) was retained, but a further lower mobility band (band 4) was now seen, and the faster band also differed in its mobility.

Further experiments were performed to see whether these bands were truly DNA-specific, and also to test the tissue-specificity (Figure 7.4). Here, rat liver extract was used and generated a single band at a position comparable to one of the "pituitary" bands. However the other two bands were not seen with this extract. The human placental extract yielded a band of similar mobility, which likewise was competed by a 50-fold molar excess of hPRL-P2, but not by a 50-fold excess of C/EBP oligonucleotide. The higher and lower mobility bands were only seen with KCl  $\geq$  75mM, whereas these complexes were not seen with the liver extract.

Finally, an experiment was performed comparing the nuclear protein extract prepared from normal human (post-mortem) pituitary with that prepared from the TSH-secreting pituitary tumour (2 $\mu$ g aliquots in each case; Figure 7.5). Unfortunately in these experiments, an apparently artefactual low-mobility band was again seen (present even with DNA alone, not shown). Nonetheless, competition studies with excess of hPRL-P2 or C/EBP oligonucleotides showed the presence of at least three specific bands with the normal pituitary extract (competed by hPRL-P2 but not by C/EBP), together with a further high-mobility band which was competed by both

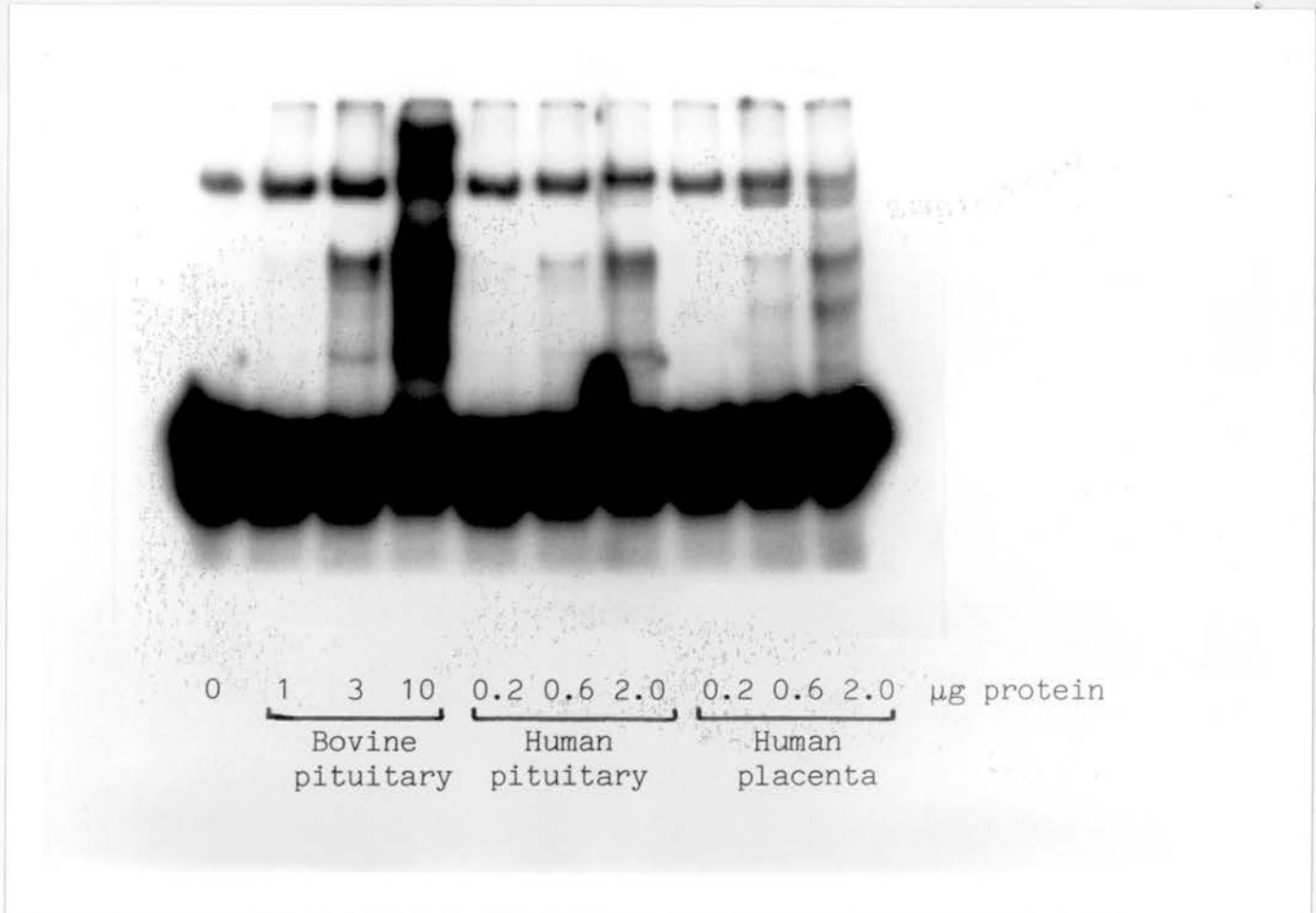


Figure 7.3

Comparison of nuclear proteins from bovine and human pituitary tissue, and human placenta. Lane 1, labelled DNA (hPRL-P2) alone; lanes 2-10, DNA incubated with varying amounts of nuclear protein as shown. Incubation carried out using 50mM KCl.

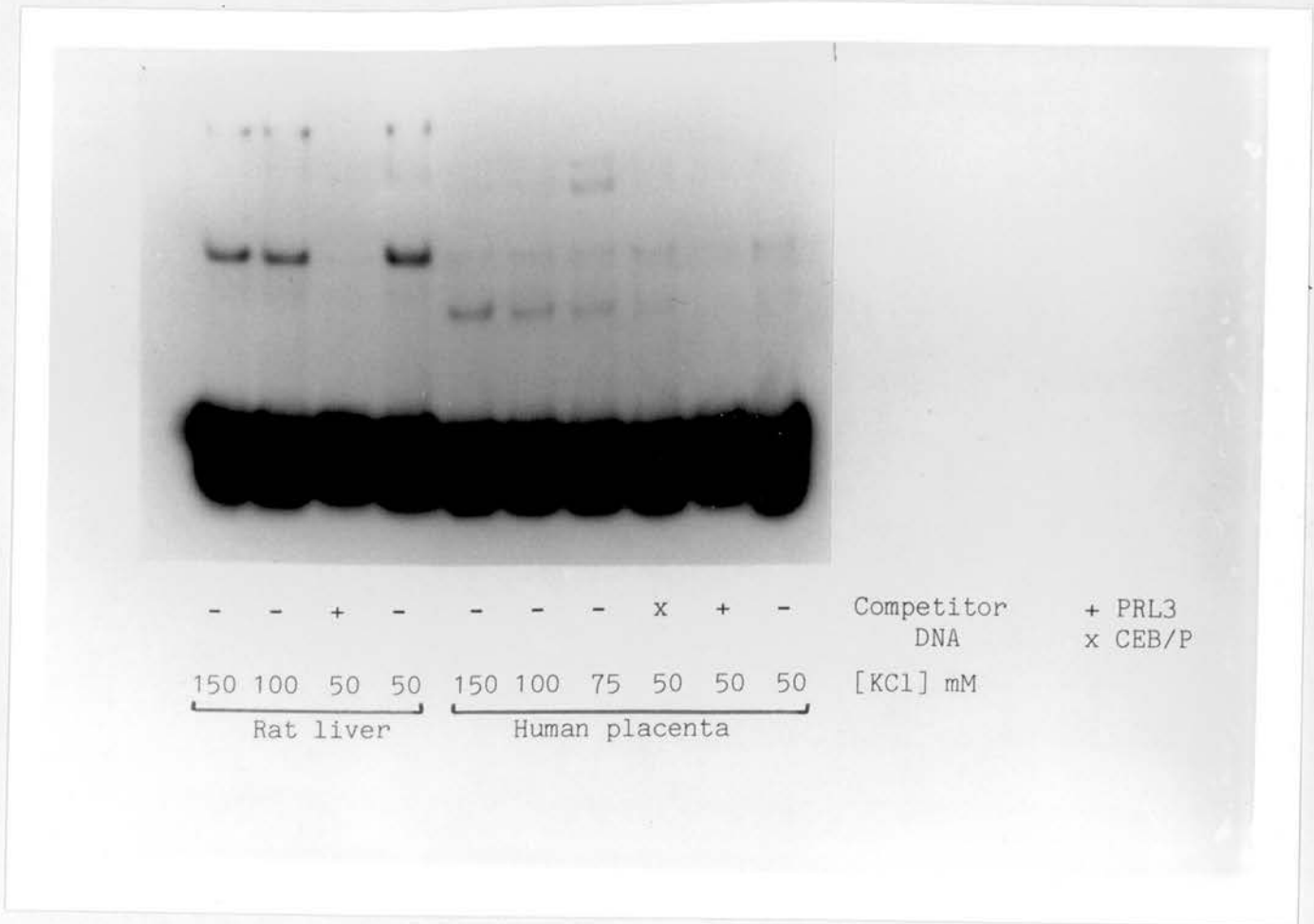


Figure 7.4

Comparison of nuclear proteins from rat liver and human placenta, with varying KCl concentrations as shown. Each lane contained 10 $\mu$ g nuclear protein, and competitor DNA (hPRL-P2, lanes 3 and 9; C/EBP, lane 8) was added at 50-fold molar excess.

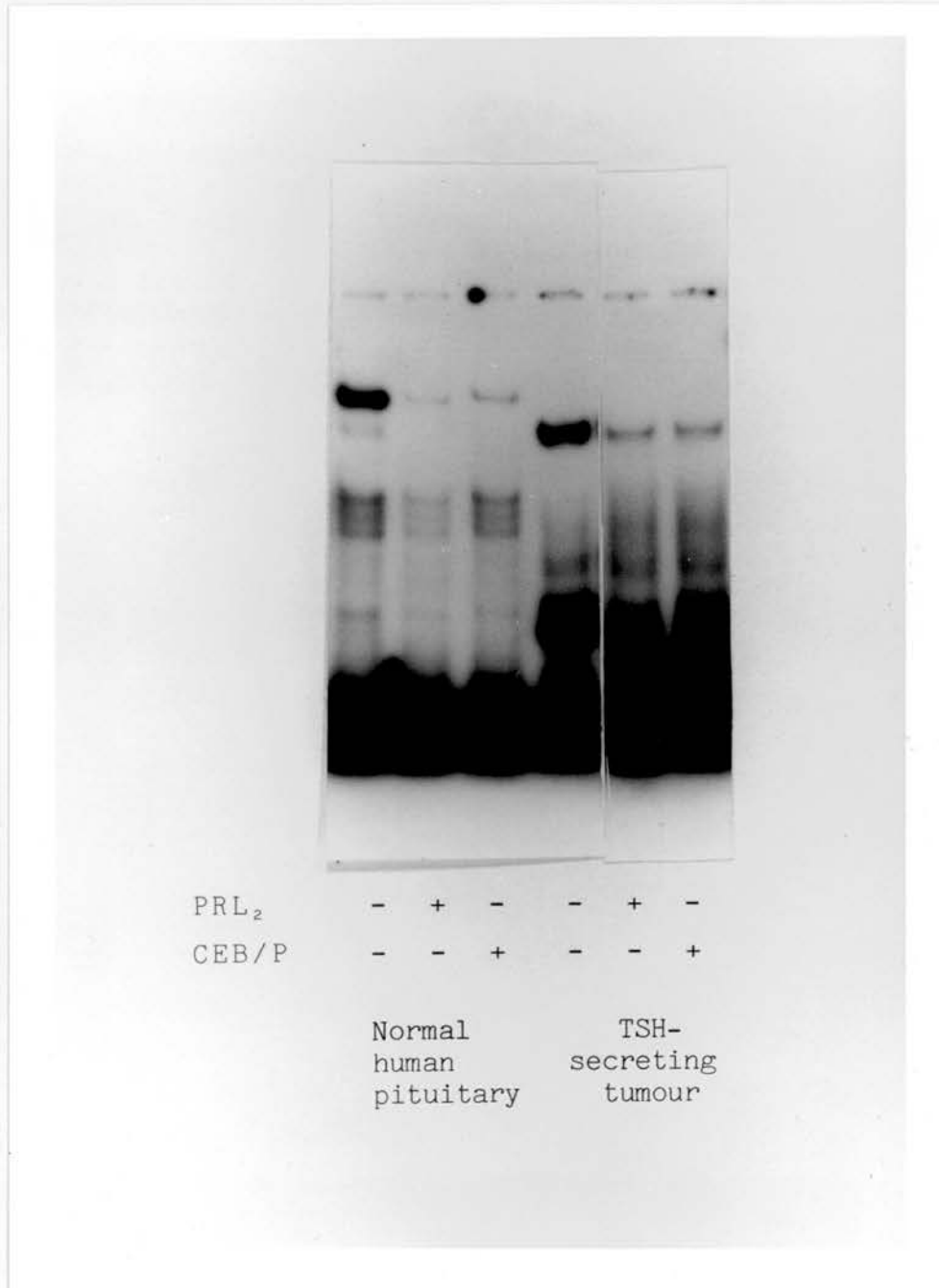


Figure 7.5

Comparison of nuclear proteins from normal post-mortem human pituitary with TSH-secreting pituitary adenoma. Each lane contains labelled hPRL-P2 oligonucleotide with 2 $\mu$ g nuclear protein, together with no competitor DNA (lanes 1 and 4), or 50-fold molar excess of hPRL-P2 (lanes 2 and 5) or C/EBP (lanes 3 and 6).



oligos. The tumour extract generated bands of quite different electrophoretic mobility from the normal pituitary, but the most prominent complex was apparently DNA-specific as it was competed out by excess hPRL-P2 and not C/EBP DNA.

#### 7.4 DISCUSSION

In this Chapter I have presented the results of preliminary experiments applying a transient transfection assay and the gel retardation assay to human pituitary tissue. The attempt at transient transfection was unsuccessful, probably mainly due to poor condition of the cells before transfection as well as their low numbers. The reason for attempting to use this approach was to address the question of why these tumour cells had apparently lost their responsiveness to dopaminergic inhibition of prolactin mRNA accumulation. One possibility would be that the 5'-flanking DNA of the endogenous hPRL gene was altered in some way that prevented dopaminergic regulation of transcription; a second possibility would be that a nuclear protein *trans*-acting factor (such as Pit-1/GHF-1) was abnormal and unable to convey an intracellular signal to regulate transcription. Thus transfection of a normal exogenous hPRL promoter sequence might enable one to distinguish between these possibilities. Transient transfection analysis has been successfully applied to primary cell culture systems (Burrin et al, 1989) and the use of the  $\beta$ -galactosidase reporter gene and the substrate X-gal (with generation of a blue reaction product visible by light microscopy; Riabowol et al, 1988) may facilitate future studies in this direction.

The gel retardation assays were designed to seek the presence of a nuclear protein similar to Pit-1/GHF-1 in human tissues which express the

PRL gene. DNase I footprinting studies have shown that a single protein factor present in the GH<sub>3</sub>B6 rat pituitary lactotroph cell line is capable of binding to DNA sequences in the 5'-flanking region of the hPRL, hGH and placental lactogen genes (Lemaigre et al, 1989). The proximal 740bp of 5'-flanking DNA from the hPRL gene contain three binding sites for this protein which have some homology with similar sites in the rPRL proximal enhancer region which bind Pit-1/GHF-1. However there has so far been no direct evidence that human pituitary cells contain a factor similar to rat Pit-1/GHF-1.

The present preliminary results are important because they are the first demonstration of a DNA-binding protein in the human pituitary gland. Human placental tissue appears to contain a similar factor, though there are some differences in the protein-DNA complexes formed. The identity of the protein(s) is not yet precisely established, but the results of competition studies suggest the presence of a Pit-1/GHF-1-like protein in that competition for protein binding was observed only with a Pit-1/GHF-1 binding site, and not with a DNA sequence which binds another non-tissue specific transcription factor (C/EBP).

There is also evidence for a DNA-binding protein factor present in the thyrotrophinoma tumour cell nuclei. The rat Pit-1/GHF-1 mRNA and protein are expressed not only in lactotrophs and somatotrophs, but also in thyrotrophic cells (Crenshaw et al, 1989; Mangalam et al, 1989). Likewise, studies of transgenic mice have shown that the presence of Pit-1/GHF-1 binding sites in a promoter sequence are able to target expression of a reporter gene to all three cell types, but not to corticotrophs or gonadotrophs (Ingraham et al, 1988). Thus it is reasonable to use the TSH-secreting pituitary tumour to seek the possible presence of a Pit-1/GHF-1

related factor. The results do imply the presence of a Pit-1/GHF-1-like nuclear protein in human thyrotrophic cells, as observed in the rat. However the gel retardation pattern differed substantially from that seen with normal pituitary tissue. The tumour tissue may be presumed to contain a relatively pure population of thyrotrophic cells, whereas the normal pituitary would contain a mixed cell population, with a predominance of lactotrophs and somatotrophs. Because of the small amounts of tumour tissue normally available it has not been possible to date to obtain sufficient prolactinoma or somatotrophinoma tissue for a similar comparison of neoplastic versus normal lactotrophs. Nonetheless, from the present preliminary data, it may be postulated either that the DNA-binding protein differs in different pituitary cell types, or that neoplastic cells may differ from normal pituitary lactotrophs. If the latter possibility is verified, it may be relevant to previous data (Chapters 3 and 4) which suggest abnormal hormonal regulation of gene expression in human pituitary tumours. Thus, the observed lack of suppression of PRL or GH mRNA levels in tumour cells by dopamine and somatostatin respectively may reflect abnormal constitutive expression due to changes in DNA-binding nuclear proteins such as Pit-1/GHF-1.

## CHAPTER 8

### CONCLUSIONS: MOLECULAR APPROACHES TO THE PATHOGENESIS OF PITUITARY TUMOURS

#### 8.1 INTRODUCTION

In this thesis, the techniques of RNA-DNA hybridization, transient transfection analysis and gel retardation analysis have been described in relation to studies of the regulation of pituitary hormone gene expression. They have been increasingly widely used in published studies of gene expression in rat pituitary cells, and two particular examples are given in Chapters 2 and 5, which concentrate on regulation of the rat prolactin gene by dopamine and calmodulin. In the other Chapters I have attempted to apply these techniques to ask questions about possible abnormalities of pituitary hormone gene transcription in human pituitary adenoma tissue. These studies are invariably circumscribed by the lack of large amounts of tissue, and the results reported here have to be regarded as somewhat preliminary. However, they indicate that it is possible to study newly developing hypotheses about the regulation of gene transcription, even in small amounts of human pituitary tissue.

#### 8.2 GENE REGULATION AND PITUITARY ADENOMA PATHOGENESIS

The basic cellular mechanisms that may result in the formation of hormone-secreting pituitary adenomas are not yet understood (Melmed et al, 1986; Melmed, 1990), but a number of recent studies of PRL and GH gene expression have suggested some potential pathological processes which may

be relevant. In particular it is interesting to note that for both PRL and GH, hormone secretion and tumour growth appear to be functionally linked, suggesting that the mechanisms for gene regulation elaborated above may have direct relevance for understanding tumour growth.

#### 8.2.1 Studies of mRNA regulation in pituitary tumours

Investigation of PRL and GH gene regulation in human pituitary tumour tissue is clearly of interest, but such studies have been very few. Yamashita et al (1986b) showed that hGH mRNA levels were appropriately inhibited by IGF-I and stimulated by GRF in human somatotroph adenomas, as has been found in normal rat pituitary tissue (Barinaga et al, 1983; Yamashita & Melmed, 1987). The present studies discussed in Chapter 4 confirmed this appropriate effect of GRF, but found that somatostatin was ineffective in suppressing GH mRNA levels in contrast to similar studies of normal rat tissue. Quantitative *in situ* hybridization analysis has also suggested a lack of suppression of GH mRNA by somatostatin in somatotroph adenomas (Levy & Lightman, 1987). In Chapter 3, the study of an aggressive prolactinoma showed resistance of hPRL mRNA accumulation to dopaminergic suppression, again suggesting that gene expression in pituitary adenomas may be relatively autonomous.

Isaacs et al (1987b) compared the expression of the endogenous hGH gene in cultured human pituitary tumour cells with the hGH gene transfected into rat cell lines, and found differences in T3 regulation, while insulin and glucocorticoid regulation was similar. Again this may imply a tumour-specific abnormality in GH gene control, but a certain answer to this question will necessitate comparison of normal human pituitary tissue with adenoma tissue. This is difficult to achieve as it is hard to obtain viable normal pituitary tissue post mortem. However the technique of

quantitative *in situ* hybridization analysis (Pixley et al, 1987; Levy & Lightman, 1988) may prove helpful in further studies of very small tissue samples such as peradenomatous tissue obtained at pituitary adenectomy.

#### 8.2.2 Hormone gene structure and proto-oncogenes

Abnormalities at the level of the hGH gene itself have been proposed by U et al (1988) who found evidence for hypomethylation of the hGH gene and its 5'-flanking region in adenomas compared to normal pituitary tissue: gene hypomethylation is generally associated with enhanced expression in tissues where the genes are normally expressed (Razin & Riggs, 1980; Compere & Palmiter, 1981; Hjelle et al, 1982; Durrin et al, 1984; Laverriere et al, 1986).

U et al (1988) also found that the *c-fos* proto-oncogene (but not *c-myc*) was amplified in genomic DNA from a single prolactinoma, which is of interest in that *c-fos* has been implicated in a mitogenic action of GRF and cyclic AMP in somatotrophs (Billestrup et al, 1987), and is closely linked to the DNA-binding and transcriptional activity of the transcription factor AP-1 (Chiu et al, 1988; Curran & Franza, 1988; Karin, 1989; Mitchell & Tjian, 1989). The role of such transcription factors in pituitary tumour development has yet to be addressed, but abnormalities in their DNA-binding or in their phosphorylation by kinases may clearly have major effects on the level of gene transcription, and may be further sites of gene dysregulation involved in adenoma formation. The recent cloning and sequencing of cDNAs coding for the pituitary-specific transcription factor Pit-1/GHF-1 (Bodner et al, 1988; Ingraham et al, 1988) will undoubtedly stimulate investigation into the potential interactions between trans-acting factors in pituitary tumours.

### 8.2.3 Abnormalities in membrane proteins: application of PCR

Abnormalities in membrane transduction proteins linked to intracellular signalling systems have been recently been found in pituitary tumours. A selective deficiency of  $G_o$  proteins has been reported in two dopamine-resistant PRL-secreting rat pituitary tumours (Collu et al, 1988). These G-proteins are thought to be involved in dopamine action, and thus their deficiency or dysfunction might lead to dopamine resistance even where dopamine receptors are present. Vallar et al (1987) reported apparent constitutive activation of adenylate cyclase activity in a sub-set of human GH-secreting adenomas, and suggested that an abnormality in the  $G_{\alpha}$  sub-unit might be responsible.

This group have recently confirmed this hypothesis using the technique of polymerase chain reaction (PCR). Complementary DNAs for the  $G_{\alpha}$  mRNA were amplified by PCR from mRNA extracted from eight GH-secreting tumours. When the amplified cDNAs were subcloned and sequenced, it was found that the  $G_{\alpha}$  sub-units contained point mutations in all four of the GH-producing tumours which demonstrated constitutively active adenylate cyclase activity but in none of the other four tumours - transfection-expression experiments using  $G_{\alpha}$  sequences containing these point mutations showed that they had the effect of increasing adenylate cyclase activity in the transfected host cells (Landis et al, 1989). Increased cAMP production results in GH secretion, increased GH gene transcription, and also increased somatotroph proliferation (Billestrup et al, 1986), and hence a "cause" of tumour formation has been found in this sub-set of somatotroph tumours.



### 8.3 CONCLUSION

The prolactin and GH genes are related and their structure and regulation have been extensively studied in rat pituitary cell systems. Mechanisms for their tissue-specific expression and hormonal regulation are rapidly being elucidated, and a complex picture of inter-related nuclear protein/proto-oncogene effects on the genes is developing. The recent identification and cloning of such trans-acting factors as Pit-1/GHF-1 and AP-1 are major advances in the field, and will undoubtedly clarify our understanding.

It seems likely that the technique of PCR will prove ideal for analysis of abnormal gene structure in small amounts of tissue in a variety of endocrine conditions where experimental data have suggested specific molecular abnormalities. In the light of the data presented in this thesis regarding pituitary tumours, it seems worthwhile to use PCR to seek mutational abnormalities in nuclear proteins that are involved in GH and PRL gene transcription: potential candidate proteins might include the oestrogen receptor and the T3 receptor, but perhaps also Pit-1/GHF-1 itself, especially in the light of recent evidence that this protein may mediate between the cAMP-CREB system, and somatotroph proliferation.

The pathogenesis of pituitary tumours remains a challenge, and has recently stimulated the application of molecular biological techniques and knowledge to human pituitary tissue. Whether tumour development can be attributed to specific defects at the level of hormone and/or proto-oncogene regulation within the nucleus will be the next exciting question that must now be addressed.



**APPENDICES**

## APPENDIX 1

### CELL CULTURE PROCEDURES

#### A1.1 PRIMARY CELL CULTURE TECHNIQUE

For all studies involving *in vitro* treatments of normal rat pituitary cells or human pituitary tumours, primary monolayer cell cultures were prepared from freshly obtained tissue. The technique was essentially a modification of that first described by Vale et al (1972), which has been widely used to study the control of secretion of different pituitary hormones. The procedure involves enzymatic and mechanical dispersion of pituitary tissue, followed by plating of dispersed cells onto multiwell plastic culture dishes. The cells are normally allowed to recover from enzymatic dispersion for at least three days before experiments commence.

#### Materials

Dulbecco's modified Eagle's medium (DMEM) was obtained in powdered form (Gibco) and diluted with distilled deionized water, adjusted to pH 7.2, and sterilized by membrane filtration. This medium was used with various supplements as follows:

<i>Initial medium (IM):</i>	DMEM +	0.3% (w/v) bovine serum albumin
		60mg/l penicillin
		100mg/l streptomycin
		1.25mg/l amphotericin
<i>Dispersion medium:</i>	IM +	0.2% (w/v) collagenase
		0.1% hyaluronidase type III
		2µg/ml DNase I

*Final medium:* IM + 10% (v/v) horse serum (Wellcome)  
2.5% fetal calf serum (Gibco)  
2mM L-glutamine

## Procedure

All glassware was siliconized ("Repelcote", Hopkin & Williams) and sterilized by autoclaving before use. For experiments using normal rat pituitary tissue (Chapter 2) male Wistar rats were anaesthetized with diethyl ether and decapitated. The brain was rapidly removed and the anterior pituitary separated from neurohypophysial tissue and placed in ice-cold Initial Medium. For experiments using human pituitary tumour tissue, fragments of tumour were obtained at trans-sphenoidal or trans-frontal hypophysectomy operations on patients with pituitary tumours, and immediately placed in ice-cold Initial Medium.

The tissue was chopped with a blade and the resulting small fragments were incubated with Dispersion Medium at 37°C in a siliconized trypsinizing chamber. Enzymatic dispersion was assisted by mechanical agitation of fragments with a magnetic stirrer bar and repeated trituration with a 1mm siliconized glass Pasteur pipette. The dispersion was usually completed within 30 min, and the cell suspension filtered through a 120µm nylon mesh to remove large undigested clumps of tissue. The filtered suspension was centrifuged at 600g for 10 min to remove enzyme solution, the pellet resuspended and washed twice more in fresh Initial Medium.

Finally, cells were resuspended in 2ml Final Medium, and a 15 $\mu$ l aliquot was diluted with 0.2% trypan blue for cell counting and assessment of viability. Average cell yield was approximately one million cells per rat pituitary gland, with over 90% viability. This cell suspension was then diluted in Final Medium to allow plating of cells onto 24-well culture plates at a density of  $1-5 \times 10^5$ .

cells in 2ml per well. The cells were allowed to attach to the plastic culture dishes and incubated in monolayer culture at 37°C in a humidified atmosphere of 95% air/5% carbon dioxide.

#### A1.2 GH<sub>3</sub> CELL CULTURE TECHNIQUE

The studies investigating calmodulin-dependence of prolactin mRNA accumulation, transient transfection analysis and gel retardation analysis were all carried out using the clonal rat pituitary GH<sub>3</sub> cell line. This cell line is one of several derivatives of the original GH cell line (Tashjian et al, 1968; Tashjian 1979) originally obtained from an oestrogen-induced rat pituitary tumour (MtT/W5; Furth et al, 1973). While GH<sub>3</sub>, GH<sub>3</sub>B6 and GH<sub>4</sub>C1 cell lines produce large quantities of prolactin as well as growth hormone, the GH<sub>1</sub> line produces mainly growth hormone, while the GC line produces no detectable prolactin. All of these lines have been well characterized in terms of the mechanisms of hormone secretion, and are widely used for the majority of detailed studies of the regulation of growth hormone or prolactin gene transcription.

GH<sub>3</sub> cells were obtained as a growing culture from the European Collection of Animal and Cell Cultures (ECACC; PHLS Centre for Applied Microbiological Research, Porton Down, Wiltshire). The cells were grown in continuous monolayer culture in antibiotic-free Ham's F-10 medium (Gibco) supplemented with 10% horse serum (Gibco, HS3, cat ref 034-6055M) and 5% fetal calf serum (Gibco, cat ref 011-06290M). In these conditions the cells had a doubling time of approximately 48h.

Cells were fed with fresh medium every 3 days and usually subcultured once weekly. In general, the cells were subcultured up to a maximum of 20 times. All manipulations of the cells were performed using disposable plastic pipettes,

and all solutions were filter-sterilised immediately before use.

#### Subculturing technique

Cells were grown to near confluence in 25cm<sup>2</sup> or 75cm<sup>2</sup> culture flasks (Falcon), and subcultured before cells began to detach. Cells never achieved complete confluence, and were usually only semi-adherent.

1. Serum-containing F-10 medium was removed, and replaced with Hanks buffered salt solution (HBSS; Gibco) to wash the cells.
2. This was aspirated and in turn replaced with 0.25% trypsin (type IX, Sigma) for 30 sec.
3. Most of this excess trypsin was aspirated, and the cells were incubated at 37°C for 5 min, covered with only a thin film of the trypsin solution.
4. Serum-containing F-10 medium was added to each flask, and the dispersed cells thoroughly resuspended; the cell suspension was then aspirated and added to fresh culture flasks. In general, cells were split 1:3 to 1:5.

#### Freezing and thawing of cells

A stock of deep frozen GH<sub>3</sub> cells was maintained in liquid nitrogen for provision of fresh cultures approximately every six months, to avoid excessive sub-culturing.

Cells were trypsinized from flasks as described above, but were resuspended in small volumes of neat fetal calf serum containing 9% dimethylsulphoxide (DMSO) as a cryopreservative agent. Approximately 10<sup>7</sup> cells per ml were then placed in cryotubes, and gradually cooled to -70°C before transfer to the liquid nitrogen bank for long-term storage.

Frozen cells were rapidly thawed as required to 37°C, and diluted into serum-containing F-10 medium, washed free of DMSO, resuspended in fresh medium and plated into 75cm<sup>2</sup> flasks.

## APPENDIX 2

### RADIOLABELLING OF DNA SEQUENCES

#### A2.1 LABELLING OF cDNA PROBES BY NICK TRANSLATION

Plasmids containing complementary DNA inserts encoding rat prolactin (Gubbins et al, 1979), rat growth hormone (Harpold et al, 1978), and human prolactin (Truong et al, 1984) were generously provided by Dr RA Maurer (University of Iowa, USA), Dr FC Bancroft (Cornell University, New York, USA), and Dr JA Martial (Université de Liège, Belgium) respectively. These cDNA probes were radio-labelled by the nick translation reaction (Kelly et al, 1970). In this reaction DNase I introduces nicks into the double stranded DNA sequence, and these nicks act as loci for DNA polymerase I to replace the original nucleotides with replicas that may be radiolabelled with  $\alpha^{32}\text{P}$ .

The reagents were purchased in the form of a kit (Amersham International, catalogue ref. N 5000), and the protocol outlined below is derived from the manufacturer's instructions. The reaction was performed on ice behind a perspex screen.

1. Reagents were added:
  - 1 $\mu\text{g}$  plasmid DNA containing insert
  - 20 $\mu\text{l}$  dNTP buffer (100 $\mu\text{M}$  dATP, dGTP & dTTP)
  - 10 $\mu\text{l}$  enzyme solution (5u DNA polymerase I and  
100pg DNase I)
  - 12.5 $\mu\text{l}$   $\alpha^{32}\text{P}$ -dCTP (=312pmol)
  - distilled deionized water to volume of 100 $\mu\text{l}$
2. The reaction mix was gently agitated, then incubated at 15°C for 90 min.

3. The reaction was terminated by adding the reaction mix to a 10cm spin column of Sephadex G-10 suspended in a buffer containing 150mM NaCl, 10mM EDTA, 0.1% SDS and 50mM Tris-HCl, pH 7.5. The column was mounted in a 10ml centrifuge tube containing the conical base of an Eppendorf 1.5ml tube, centrifuged at 1000rpm for 5 min, and the labelled DNA collected in the eluate.
4. Incorporation was calculated as follows:
  - a. a 2 $\mu$ l aliquot of total reaction mix was added to 200 $\mu$ l water (= total counts)
  - b. 20 $\mu$ l of this solution was added to 50 $\mu$ l carrier DNA (salmon sperm) solution; 1ml 10% trichloroacetic acid (TCA) solution added; mixture incubated on ice for 15 min, to allow labelled cDNA and carrier DNA to precipitate. Precipitated DNA collected onto glass-fibre disc by vacuum filtration; disc washed with fresh TCA, dried, and counted by liquid scintillation counting.
  - c. 20 $\mu$ l of total counts solution added to glass-fibre disc for counting.
  - d. 20 $\mu$ l of solution eluted from Sephadex column added to glass-fibre disc and counted.
  - e. 20 $\mu$ l of column eluate TCA-precipitated and counted.

$$\% \text{ incorporation} = \frac{\text{dpm on filter}}{\text{total activity in dpm}} \times 100\%$$

$$\text{Specific activity} = \text{dpm on filter} \times \frac{202}{20} \times \frac{100}{2} \text{ dpm}/\mu\text{g.}$$

Mean specific activity was  $1-4 \times 10^8$  dpm/ $\mu$ g DNA.

5. Before use in a hybridization reaction, the labelled probe was denatured by heating to 100°C and snap cooling on ice.

## A2.2 LABELLING OF hGH cDNA BY RANDOM PRIMER TECHNIQUE

#### A2.2.1 Excision of hGH cDNA insert

The hGH cDNA is an 800bp insert within pBR322, and can be excised using HindIII restriction enzyme before radiolabelling.

Buffer: 5x Tris-Borate-EDTA (TBE) 54g Trizma base  
27.5g boric acid  
20ml 0.5M EDTA, pH8.0  
make up to 1 litre with dH<sub>2</sub>O

Loading buffer	50mM Tris
	50mM EDTA
	0.5% SDS
	0.1% bromophenol blue
	12.5% sucrose

1. 4 $\mu$ l of DNA (1 $\mu$ g/ $\mu$ l) added to 2 $\mu$ l enzyme with 2 $\mu$ l buffer (as recommended by manufacturer), and made up to 20 $\mu$ l on ice.
2. Reaction mix incubated 4-16h at 37°C
3. Gel prepared: low melting point agarose (1% in 25mM (1x) TBE buffer) boiled to dissolve agarose, and ethidium bromide added to stain DNA bands.
4. Gel poured and allowed to set.
5. Loading buffer (2 $\mu$ l) added to cDNA and  $\lambda$  HindIII DNA markers.
6. Electrophoresis tank filled with 1x TBE. Samples loaded into wells and run at 60v (neg to pos) for 1-2h.



7. After electrophoresis, bands visualised under UV light, and band with insert cDNA cut out with a scalpel blade, weighed (in Eppendorf tube) and water added (1.5µl/mg agarose). Agarose boiled for 7 min, cooled to 37°C, and aliquots prepared for storage until required for labelling.

#### A2.2.2 Radiolabelling of excised cDNA probe

This technique relies on the use of random DNA hexamers that are able to bind to sequences within a denatured cDNA probe, and act as primers to allow initiation of transcription from the other strand by DNA polymerase (Feinberg & Vogelstein, 1983). The reagents were used in kit form (Amersham, RPN 1601) and the protocol outlined below is essentially as recommended in the manufacturer's instructions.

1. cDNA probe (25ng) was heat denatured by heating to 100°C for 2-3 min, and snap-cooled on ice.

2. Reagents were added to cDNA:

Multiprime buffer solution	11µl
Primer solution	5µl
$\alpha^{32}\text{P}$ -dCTP (50µCi)	5µl
Water to final volume	50µl
Enzyme solution	2µl

*Multiprime buffer solution:*

*dATP, dGTP, dTTP in buffer containing Tris-HCl pH 7.8, MgCl<sub>2</sub> and 2-mercaptoethanol*

*Primer solution contained:*

*random hexanucleotides in BSA solution*

*Enzyme solution contained:*

*DNA polymerase I, Klenow fragment (1u/µl) in 50mM potassium phosphate (pH 6.5), 10mM 2-mercaptoethanol and 50% glycerol*

3. The reaction mix was gently mixed by pipetting, avoiding vortexing, and was then incubated at room temperature for 3-24h.
4. Incorporation was checked by comparing TCA-precipitable counts from the reaction mix with total counts, similarly to the procedure described for nick translation in A2.1. Mean specific activity was approximately  $1 \times 10^9$  dpm/ $\mu$ g DNA.
5. Before use in a hybridization reaction, the labelled probe was denatured by heating to 100°C and snap cooling on ice.

#### A2.3 LABELLING OF OLIGONUCLEOTIDES WITH T4 POLYNUCLEOTIDE KINASE

Double stranded oligonucleotides may be end-labelled for use in gel retardation assays using T4 polynucleotide kinase. This enzyme was first purified from *Escherichia coli* infected with the T4 bacteriophage. In the "forward reaction" (more widely used than the less efficient exchange reaction), the enzyme catalyses the transfer of the terminal ( $\gamma$ ) phosphate from ATP to the 5' hydroxyl terminus of DNA or RNA. The 5' end of a DNA sequence may be radiolabelled whether it is recessed, overhanging or blunt-ended.

In a 30 $\mu$ l reaction, reagents were added as follows:

1. 50mM Tris-HCl, pH 7.5  
10mM MgCl<sub>2</sub>  
5mM DTT  
1-50pmol dephosphorylated DNA, 5' ends  
50pmol (150 $\mu$ Ci)  $\gamma$ -<sup>32</sup>P-ATP (>3000 Ci/mmol)

50µg/ml bovine serum albumin

20 units T4 polynucleotide kinase

2. The reaction mix was incubated at 37°C for 60 min, and terminated by addition of 1µl 0.5M EDTA. If desired, labelled DNA could be separated from unincorporated nucleotide on a Sephadex G-100 column, or a G-50 spin column.

## APPENDIX 3

### HYBRIDIZATION TECHNIQUES

#### A3.1 CYTOPLASMIC DOT HYBRIDIZATION ASSAY (White & Bancroft, 1982)

##### A3.1.1 Preparation of cytoplasmic samples

1. Medium was removed from cell culture wells for hormone assay, and cells were scraped from the plastic surface in ice-cold phosphate buffered saline (PBS), and centrifuged at 600g for 10 min.
2. Cells were resuspended in 1ml PBS, and recentrifuged at 15000g for 30s.
3. Cells were resuspended on ice in 45µl Tris (10mM) with 1mM EDTA, pH 7.0.
4. Two 5µl aliquots of 5% Nonidet P40 were added, with mixing on ice for 5 min in between. [This step is designed to cause lysis of predominantly the cytoplasmic membrane, leaving the cell nuclei intact, which can be verified microscopically.]
5. The lysed cells were recentrifuged at 15000g for 3 min, to pellet the nuclei.
6. The cytoplasmic supernatant (50µl) was added to sterile Eppendorf tubes containing 20µl 37% formaldehyde and 30µl 20x standard saline citrate (SSC) buffer [3M NaCl and 0.3M trisodium citrate, pH 7.0].
7. The final sample was heated at 60°C for 15 min, before storing at -70°C.

##### A3.1.2 Hybridization assay

The cytoplasmic samples were serially diluted (1:10 to 1:100) in 15x SSC, and 100µl of each dilution was applied in duplicate with suction to a nitrocellulose or nylon hybridization transfer membrane held in a 96-well perspex manifold ("Hybridot" apparatus, BRL). The membrane was baked at 80°C for 90 to fix RNA to the membrane.

### Prehybridization

The membrane was prehybridized, to block DNA binding to non-specific sites on the membrane, by incubation at 42°C for 16-24h in a polythene bag with approximately 10ml of a buffer containing:

- 50% (v/v) deionized formamide
- 0.2% (w/v) polyvinyl pyrrolidone
- 0.2% (w/v) bovine serum albumin
- 0.2% (w/v) ficoll
- 0.05M Tris-HCl, pH 7.5
- 0.1% (w/v) sodium pyrophosphate
- 1% (w/v) sodium dodecyl sulphate (SDS)
- 10% (w/v) dextran sulphate
- 100µg/ml denatured salmon sperm DNA
- 5.8% (w/v) NaCl

### Hybridization

The <sup>32</sup>P-labelled cDNA probe (approx 250ng) was denatured by heating at 100°C for 10 min then snap cooling on ice, diluted in approximately 2ml of prehybridization buffer and then added to the prehybridization buffer surrounding the membrane. The membrane was gently agitated in the hybridization buffer for 16h at 42°C, using a shaking water bath.

### Washing

After hybridization, the membrane was removed from the buffer containing labelled probe, and was washed as follows:

- Twice in: 2x SSC, 5 min, room temperature;
- Twice in: 2x SSC with 1% SDS, 45 min, 65°C;
- Twice in: 0.1x SSC, 30 min, room temperature

[SSC buffers were prepared by dilution of 20x SSC stock solution.]

Autoradiography

Membranes were air-dried and then exposed to X-ray film (Cronex-4, NIF100) using intensifying screens at -70°C for up to 72h. Intensity of hybridization was measured by scanning densitometry of the developed autoradiographs using a laser densitometer (LKB 2202 Ultrosan) to generate optical density estimates for each dot. The optical density signal was non-linear at the extremes of the range, and exposure of autoradiographs therefore had to be judged to give a signal which would yield results in the linear part of the range for at least one of the cytoplasmic dilutions used.

### A3.2 NORTHERN BLOT ANALYSIS

#### A3.2.1 RNA preparation: guanidine isothiocyanate/caesium chloride method

Total RNA was extracted from cultured human somatotrophinoma cells using the technique described by Chirgwin et al (1979) for tissues enriched in ribonucleases. The total RNA (including nuclear and cytoplasmic RNA is relatively pure, uncontaminated by proteins. The technique relies on the fact that RNA sediments more rapidly through a caesium chloride cushion than DNA or protein; guanidine isothiocyanate (GI) is used to lyse cells and inactivate ribonucleases simultaneously. Caution is required to ensure that there is no nuclease contamination from skin or solutions, and all glassware should be treated either by baking (800°C for at least 4h) or by exposure to diethylpyrocarbonate solution (DEPC).

#### Solutions

4M guanidine isothiocyanate:	Guanidine isothiocyanate	50g
	Na-N laurylsarcosine	0.5g
	1M Na citrate pH 7.4	2.5ml
	Surgine antifoam A	0.33ml
	Distilled water to 100ml	

Filter under reduced pressure, and then add

0.7ml  $\beta$ -mercaptoethanol.

5.7M CsCl in 0.1M EDTA:	Caesium chloride, 96g/100ml
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### Method

1. Add GI solution to cells in culture wells, and make this lysate up to 3ml with further GI solution, resuspending thoroughly to ensure cell disruption.
2. Add solid CsCl to the homogenate, 1.2g CsCl per 3ml GI solution and gently shake to dissolve.
3. Layer cell lysate onto 1.2ml cushion of 5.7M CsCl/EDTA in a 5ml polyallomer ultracentrifuge tube. Top up tubes with extra GI/CsCl solution if necessary, and balance tubes.
4. Centrifuge in SW50.1 rotor at 35000 rpm for 17h.
5. Carefully remove GI/CsCl from tubes, without disturbing the RNA pellet.
6. Thoroughly resuspend the pellet in 100 $\mu$ l distilled water, and transfer the RNA solution to a sterile Eppendorf tube.
7. Phenol extract the RNA to remove any protein: add equal volume of phenol, vortex for 20s, and spin for 5 min in a microcentrifuge (13000rpm). Transfer upper aqueous layer to fresh Eppendorf tube, and remove residual phenol by adding 0.5ml water saturated ether. Shake gently, then spin for 5 min, remove upper layer of ether and dry off remaining ether by heating RNA solution at 50°C for 5 min.
8. Ethanol precipitate RNA: add 1/10th volume of 3M Na acetate, pH 5.2 and mix; then add 2.5 volumes absolute ethanol and mix. Precipitate RNA by leaving tubes at -70°C for 2h or -20°C for 16h.
9. Pellet RNA by spinning for 10 min at 4°C in a microcentrifuge; a small white pellet should be visible. Remove supernatant, and add 250 $\mu$ l cold 70% ethanol. Keep at -20°C for 10 min, then re-centrifuge for 5 min. Remove ethanol, and dry pellet in a vacuum evaporator. The RNA may be resuspended in 10 $\mu$ l sterile water and stored at -70°C.



10. RNA content and purity is estimated by diluting in water and reading optical density at 260nm and 280nm. At 260nm, one OD unit = 40µg/ml. The OD260/OD280 ratio should approach 2.0 if the RNA is uncontaminated by protein or phenol.

#### A3.2.2 Agarose gel electrophoresis and Northern blotting

RNA species may be separated by electrophoresis through an agarose gel; the percentage of agarose may be varied according to the size of RNA species sought. For analysis of hGH mRNA, a 1.0% agarose gel was used. The blotting procedure transfers RNA onto a nitrocellulose membrane where they are fixed by baking before hybridization is performed as described in section A3.1.2.

#### Solutions

0.25M phosphate buffer, pH 6.5	17.75g $\text{Na}_2\text{HPO}_4$
	19.5g $\text{NaH}_2\text{PO}_4$
	add distilled water to 1 litre

6M glyoxal	Obtained as 6M solution, but should be deionised by passage through a mixed bed resin (Bio-Rad AG501-X8) until the pH is neutral; store at -20°C in small aliquots in tightly sealed tubes.
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Loading buffer	50% glycerol
	0.01M phosphate buffer
	0.4% bromophenol blue

Method

1. For each RNA sample, mix:

6M glyoxal	2.7 $\mu$ l
DMSO	8.0 $\mu$ l
0.1M phosphate	1.6 $\mu$ l
RNA up to 20 $\mu$ g	3.7 $\mu$ l make up with water.
2. Incubate RNA at 50°C for 60 min in tightly capped tube. For standards, use an RNA marker ladder, or a total cellular RNA sample for ethidium bromide staining to locate position of 28S and 18S ribosomal RNA. These should be glyoxalated with the RNA samples.
3. Meanwhile pour agarose gel:

For a 1% gel add 2.0g agarose to 200ml 0.01M phosphate buffer. Boil to dissolve agarose, then cool to approximately 50°C before pouring. Position comb before pouring the gel, and leave the comb in place until the gel is quite cold. Add 0.01M phosphate buffer to the electrophoresis tank to submerge gel.
4. Cool RNA samples and standards to room temperature, and add 4 $\mu$ l sterile loading buffer. Load samples and standards into wells immediately.
5. Run gel at 3-4 volts/cm for 4-5h. Recirculate buffer to maintain pH at a constant level using a peristaltic pump, once the marker dye has penetrated the gel.
6. Cut off standard track and stain with 1 $\mu$ g/ml ethidium bromide. Keep rest of the gel for Northern transfer.
7. Cut wicks of Whatman 3mm paper, and wet in 25mM phosphate buffer before putting in tank. Cut nitrocellulose membrane to the same size as the gel, and place over the wicks avoiding air bubbles. Place 5-8 sheets of Whatman paper on the gel and then place blotting pads with a heavy

weight on top. Leave overnight to allow RNA transfer onto the nitrocellulose.

8. After transfer wash membrane in 25mM phosphate buffer, air dry, and finally bake at 80°C for 2h. Finally, prehybridize, hybridize and wash the membrane as described in section A3.1.2.

## APPENDIX 4

### PROLACTIN AND GROWTH HORMONE RADIOIMMUNOASSAYS

#### A4.1 PROLACTIN RADIOIMMUNOASSAY

Prolactin secretion by cultured rat pituitary cells was assessed by double antibody radioimmunoassay, using reagents provided by the US National Hormone and Pituitary Program, NIADDK, Bethesda, Maryland, USA.

##### A4.1.1 Buffers and materials

###### Buffers

0.5M phosphate buffer:                      0.5M  $\text{NaH}_2\text{PO}_4$ , 0.5M  $\text{Na}_2\text{HPO}_4$  in  $\text{dH}_2\text{O}$ ,  
pH 7.4

0.01M PBS:                                      0.01M  $\text{NaH}_2\text{PO}_4$ , 0.01M  $\text{Na}_2\text{HPO}_4$ , 0.15M NaCl  
in  $\text{dH}_2\text{O}$ , pH 7.4

Assay buffer:                                      PBS  
+ 0.1% (w/v) EDTA  
+ 0.1% (v/v) normal rabbit serum  
+ 2% (w/v) BSA

###### Reagents

rPRL standards were prepared from rPRL-RP3 (NIADDK, reference preparation 3) which was diluted in PBS + 1% BSA, and stock solution stored in aliquots of 10 $\mu\text{g}$ /100 $\mu\text{l}$  for up to 6 months. Standards (0.78-100ng/ml) were prepared by dilution in culture medium (DMEM + 0.3% BSA) at the time of assay.

rPRL antiserum (rabbit: NIADDK-anti-rPRL-S-9) was stored in assay buffer, and diluted to a final concentration of 1/4000 for addition to assay tubes (tube concentration 1/12000).

Prolactin for iodination (NIADDK rPRL-I-5) was stored in 0.01M NaHCO<sub>3</sub> in aliquots of 2.5µg/25µl in siliconized glass Durham tubes for up to 6 months. A 15 x 1cm Sephadex G75 column was prepared and primed with PBS + 1% BSA. Reagents were added as follows:

rPRL-I-5 (2.5µg)	25µl
0.05M phosphate buffer	25µl
Na <sup>125</sup> I (1mCi)	10µl
Chloramine T (2.5mg/ml)	10µl

These reagents were gently mixed for 40s, and the reaction terminated by adding 25µl sodium metabisulphite (2mg/ml in 0.05M phosphate buffer). Reaction products were separated on the Sephadex column and fractions counted to assess incorporation (mean approximately 60%) and specific activity (mean approximately 250µCi/µg). Iodinated rPRL was diluted in assay buffer to provide 10000-15000 cpm/100µl for use in assays.

Sheep ant-rabbit IgG second antibody (Z-930, BDS Biologicals, Birmingham University) was diluted in PBS to 1/80 (tube dilution 1/320).

#### A4.1.2 Assay procedure

The assay was performed at room temperature as follows:

Day 1.	rPRL standard or sample	100µl
	Antiserum	100µl
	<sup>125</sup> I-rPRL	100µl
Day 2.	Second antibody	100µl

Day 3. Tubes centrifuged at 1720g at 4°C for 45min. Supernatant discarded and precipitate counted for bound radioactivity.

Non-specific binding (%NSB/total counts) and zero standard binding (%Bo/total counts) were calculated, and a standard curve was constructed, using a programmable calculator with a program developed by Rodbard & Lewald (1970). Assay sensitivity was approximately 1ng/ml, and intra- and inter-assay coefficients of variation at 9ng/ml were 6% and 10%.

#### A4.2 GROWTH HORMONE RADIOIMMUNOASSAY

Rat growth hormone (GH) was assayed using a fractional precipitation radioimmunoassay with reagents supplied by the US National Hormone and Pituitary Program, as for rPRL.

##### A4.2.1 Buffers and reagents

Buffers were used as described for the rPRL assay, with the exception of the assay buffer, which consisted of 0.01M PBS + 1% BSA.

rGH standards were prepared from rGH-RP2 (NIADDK reference preparation 2), stored in 25µl aliquots of a stock solution of 10µg/ml in assay buffer. Standards (0.78-100ng/ml) were diluted in assay buffer as required.

rGH antiserum (monkey: NIADDK-anti-rGH-S-4) was diluted in assay buffer to 1/3000 for addition to assay tubes (tube dilution 1/30000).

rGH label (NIADDK-rGH-I-5) was iodinated using the chloramine T method as described for rPRL. Aliquots of rGH for iodination were stored at 5µg/10µl concentrations, otherwise as for rPRL. The iodination reaction was performed exactly as described for rPRL except that volumes of rGH and 0.5M phosphate buffer were 10µl not 25µl, thus the final reaction volume was only 40µl.

A4.2.2 Assay procedure

The assay was performed at room temperature, as follows:

Day 1.	rGH standard or sample	100 $\mu$ l
	Antiserum	100 $\mu$ l
	<sup>125</sup> I-rGH	100 $\mu$ l
	Assay buffer	600 $\mu$ l

Day 2.	$\gamma$ -globulin 1% in PBS	100 $\mu$ l
	25% polyethylene glycol in PBS	1ml

Tubes were mixed and incubated for 2h before centrifuging at 1720g at 4°C for 45min. Supernatant was discarded and the precipitate counted for bound radioactivity.

Assay data and rGH concentrations were calculated as described for the rPRL assay. Assay sensitivity was approximately 1ng/ml, and intra- and inter-assay coefficients of variation were less than 10%.

### A5.1 PROTEIN ASSAY: LOWRY TECHNIQUE

### A5.1.1 Materials

Alkaline copper reagent was prepared freshly before the assay by adding reagents as follows:

1% sodium tartrate with 0.5% CuSO<sub>4</sub> 1ml

Reagents were mixed together immediately.

#### A5.1.2 Assay procedure

Reagents were added as follows:

Alkaline copper reagent                      2ml

Tubes were mixed and left to stand for 10min.

Folin's reagent	100μl
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Tubes were mixed and left to stand for at least 30min.

The optical density (750nm) of the samples was measured, and protein concentration calculated by comparison with the BSA standards.



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## **Prolactin and growth hormone**

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Abnormalities of the secretion of prolactin (PRL) or growth hormone (GH) are important and relatively common problems in clinical endocrinology and have prompted much clinical and basic research. In the past 10 years knowledge of the control of the expression of these genes has grown enormously and the fruits of this new understanding are just beginning to be applied more directly to the study of human disease. The production of recombinant human growth hormone in bacteria has had a direct impact on patient management but major advances have recently been made in the understanding of basic cellular control mechanisms in normal and neoplastic pituitary tissue and these will almost certainly influence therapeutic strategies in the future.

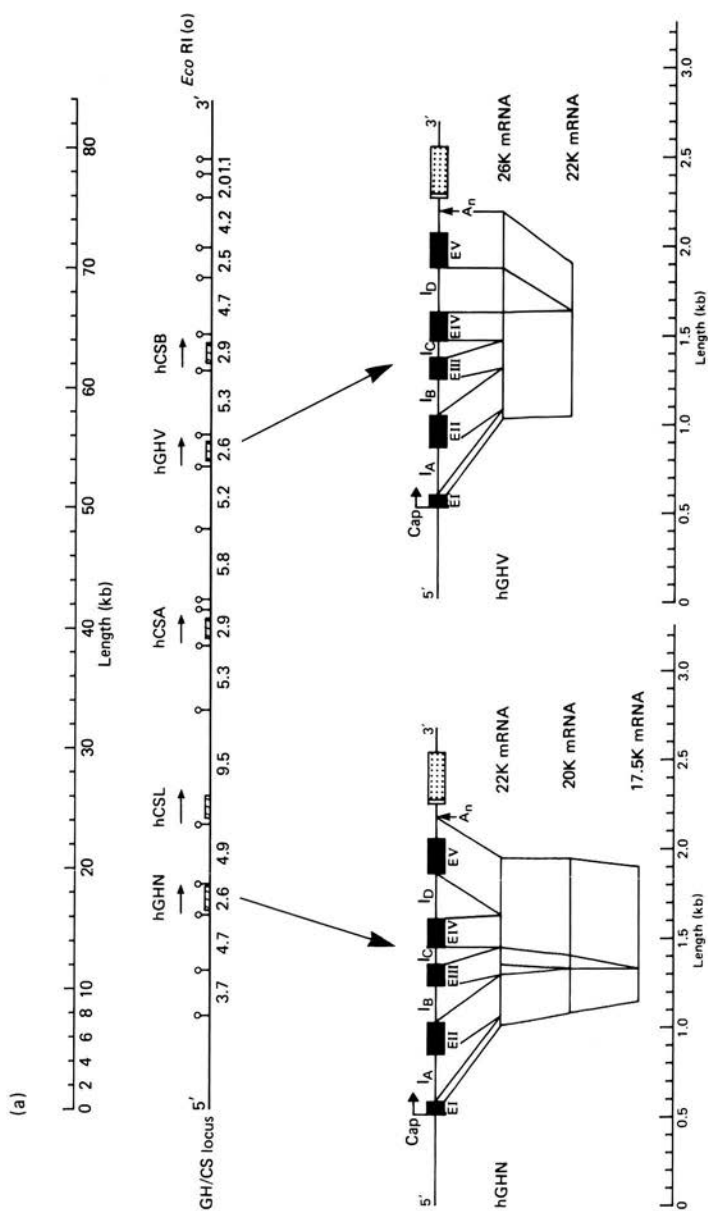
This chapter will present a brief description of the PRL-GH gene family, followed by a review of our present understanding of tissue specificity and hormonal regulation in the control of gene transcription. Finally, the principles of the application of such studies to human disease will be illustrated by some recent examples.

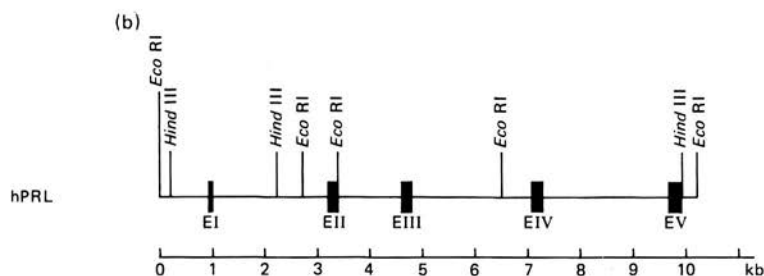
### **THE PROLACTIN AND GROWTH HORMONE GENE FAMILY**

#### **Gene structure and relationships**

The genes coding for PRL and GH are related and form part of a family which also comprises chorionic somatomammotrophin (CS, or placental lactogen, PL; reviewed in Miller and Eberhardt, 1983).<sup>\*</sup> In the rat there is a single GH gene (Barta et al, 1981), a single rPRL gene (Cooke and Baxter, 1982) and several rCS/PL genes (Duckworth et al, 1986). In man, while the single human (h)PRL gene (Cooke et al, 1981; Truong et al, 1984) is located on chromosome 6 (Owerbach et al, 1981), there is a cluster of related hGH and hCS genes (Martial et al, 1979; Seeburg, 1982) grouped together on

<sup>\*</sup> They all have five exons and the GH/CS genes differ mainly in the size of the four introns from the more distantly related PRL genes. However, the trout GH gene comprises a fifth intron interrupting the fifth exon described in all mammals (Agellon et al, 1988).





**Figure 1.** (a) Arrangement of human GH/CS gene cluster, with scales shown in kilobases (kb), *Eco* RI restriction sites marked by open circles. Possible alternative splicing of hGH-N and hGH-V genes is shown, with generation of mRNAs of differing sizes. E denotes exons (shown as black boxes) and I denotes introns;  $A_n$  denotes the polyadenylation signal. (b) Structure of human PRL gene with scale in kb and exons indicated as black boxes.

chromosome 17 (Owerbach et al, 1980; George et al, 1981; Kidd and Saunders, 1982; Hirt et al, 1987), as shown in Figure 1. The single hGH-N gene is expressed primarily in the pituitary and will be considered in more detail in this review. Alternative splicings of the hGH-N nuclear pre-mRNA (or hGH-N gene primary transcript) (DeNoto et al, 1981) have been shown to generate multiple mRNAs in the pituitary gland, one of which leads to the production of 20 kDa variant protein (Lewis et al, 1978) which constitutes 5–10% of pituitary GH, and another would code for a novel 17.5 kDa protein (Lecomte et al, 1987). The variant GH gene, hGH-V, was shown to be functional when linked to a viral promoter and transfected into monkey kidney cells in vitro (Pavlakakis et al, 1981); however, the endogenous human mRNA was only recently found in placental tissue and in very small amounts in a pituitary adenoma (Frankenne et al, 1987). The structure of hGH-V mRNA has recently been determined from a human placental cDNA library and two different mRNAs appear to be generated by alternative splicing of the pre-mRNA, giving rise to two distinct proteins (Cooke et al, 1988). The hCS-L gene is thought to be non-functional, but hCS-A and hCS-B are expressed in syncytiotrophoblast tissue (McWilliams and Boime, 1980), where they lead to the production of hCS with identical amino acid sequences (Barrera-Saldaña et al, 1983).

Among the human genes there is considerable sequence homology, up to 92% between the hGH and hCS genes (Cooke et al, 1980, 1981; Barsh et al, 1983), and 42% between the hPRL and hGH coding sequences (Martial et al, 1979; Seeburg, 1982; Truong et al, 1984). Indeed, between species there is significant homology of coding sequences, 73% between human and rat PRL and 75% between human and rat GH (Cooke et al, 1981). It has been calculated from data on the conservation of coding sequences and splicing sites that the PRL and GH genes derived from a single primordial gene and began to diverge about 400 million years ago, the period when fish and quadrupeds started to diverge (Niall et al, 1971; Cooke et al, 1981; Cooke and Baxter, 1982). Interestingly, the proximal regulatory 5'-flanking DNA sequences are also highly conserved between the rat, bovine and human PRL genes (Cooke and Baxter, 1982; Camper et al, 1984; Truong et al,

1984), particularly in regions thought to be important for tissue-specific gene expression.

Further members of the PRL gene family have recently been described in other tissues. Distinct from the placental lactogen genes, 'proliferin' has been identified from a library of DNA fragments complementary to mouse mRNA sequences (cDNA library), and appears to be linked to cellular proliferation (Linzer and Nathans, 1984; Linzer et al, 1985; Lee and Nathans, 1987); a placental 'proliferin-related protein' cDNA and a third prolactin-like protein and gene have also been described (Linzer and Nathans, 1985; Colosi et al, 1988; Duckworth et al, 1988). These genes map to chromosome 13 in the mouse, along with PRL itself (Jackson-Grusby et al, 1988) and show some degree of sequence homology with PRL, but their tissue specificity differs. PRL itself is synthesized at low levels in non-pituitary tissue: its production is hormonally regulated in human decidual tissue (Handwerger et al, 1987; Golander et al, 1988), and PRL mRNA has been detected in human decidual cells by hybridization analysis (Clements et al, 1983; Taii et al, 1984). The human decidual PRL cDNA has been cloned and the sequence found to be almost identical to that of the pituitary cDNA, confirming that there is only a single copy of the hPRL gene per haploid genome (Takahashi et al, 1984). Finally, prolactin mRNA and protein production occurs at low levels in human IM-9 lymphocytic cells, but whether this may imply some latent biological function remains to be seen, as normal human lymphocytes have no detectable prolactin mRNA (DiMattia et al, 1988).

### Ontogeny

Despite the close structural similarities between their genes, PRL and GH exhibit quite distinct developmental patterns and tissue-specific expression. PRL and GH are produced in separate cell types within the pituitary gland, lactotrophs and somatotrophs, and immunocytochemical studies suggest that they are expressed in the pituitary only late in fetal development (Gash et al, 1982). However, rat PRL and GH may be co-expressed in the same cell, and such somatomammotrophs may predominate in the late fetal pituitary, according to an immunocytochemical study (Hoeffler et al, 1985). In the rat, comparison of immunocytochemical studies with *in situ* hybridization using the rat PRL cDNA as a probe have recently shown that PRL mRNA accumulates well before PRL can be detected by immunostaining; as PRL is secreted in fetal rats, this indicates that it is synthesized but not stored in the fetal pituitary, possibly reflecting lack of fetal hypothalamic dopamine production (Hooghe-Peters et al, 1988).

Co-production of PRL and GH has been shown in adult pituitary tissue, both by immunocytochemical studies and also by reverse haemolytic plaque assays (Frawley et al, 1985; Nikitovich-Winer et al, 1987). The potential for co-expression of the PRL and GH genes in a single cell type has suggested that tissue-specific expression of these genes may be at least partly controlled by a single common factor (Nelson et al, 1988), and experimental support for this hypothesis will be discussed in detail below.

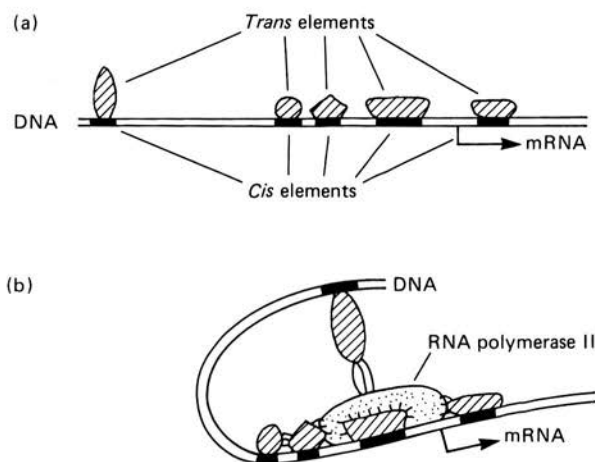
### Prolactin and GH receptors

In 1987 the amino acid sequences of the rabbit and human GH receptors were deduced from their cDNAs but no homology of the GH receptor was found with any other reported protein (Leung et al, 1987). Subsequently the rat liver PRL receptor has also been cloned (Boutin et al, 1988); this again does not resemble other known proteins but it does possess striking similarities to the GH receptor. Both receptors have large extracellular (hormone-binding) regions, a single transmembrane segment and a large cytoplasmic domain. Overall, there is 30% amino acid sequence homology but there are localized regions of sequence identity between the two proteins. The linear arrangement of these strongly homologous segments is conserved, suggesting functional importance (Boutin et al, 1988). The strong similarities between the receptors for PRL and GH recall the similarities of the hormones themselves, and suggest the possibility of a similar family of receptor genes perhaps diverging from a single ancestral gene at a similar time to PRL and GH themselves.

### TRANSCRIPTIONAL REGULATION

The regulation of gene transcription is currently considered to be exerted by the specific interaction of regulatory proteins (*trans* factors) with given DNA sequences (*cis* elements). Different types of *cis/trans* coupling are thought to allow both tissue-specific expression and also hormonal induction or inhibition of gene transcription. The *cis* regulatory sequences can be located within promoter regions, or may be part of an 'enhancer' that can regulate transcription at any distance inside or outside the gene, upstream or downstream from its promoter. Changes in the three-dimensional structure of DNA allows the DNA-bound *trans* factors to interact with the transcription initiation complex (as shown schematically in Figure 2; for reviews see Dynan and Tjian, 1985; Ptashne, 1986, 1988; Maniatis et al, 1987). Several *trans* factors have been identified (some of which correspond to cellular proto-oncogenes), including steroid and thyroid hormone receptors (see Evans, 1988, for review) and activator proteins such as AP-1 and AP-2 which are thought to mediate the effects of intracellular messenger systems (Imagawa et al, 1987; Lee et al, 1987).

The regulation of PRL and GH gene transcription involves two aspects: tissue-specific expression and hormonal regulation. Although these will be considered separately, the final level of transcriptional activity presumably reflects integration of both systems, and indeed it seems likely that their mechanisms overlap to a great extent. A general approach to understanding these mechanisms should involve (1) the identification of *cis* elements in a given gene; (2) precise definition of *cis/trans* interactions; that is, demonstration of protein-DNA binding and identification of *trans*-acting factors involved; and (3) purification and cloning of *trans*-acting factors, and assessment of their regulation. Such an approach has been used with great success in the case of the PRL and GH genes, for example with the definition of



**Figure 2.** Possible interaction of *cis* and *trans* elements. (a) *Cis* elements in the gene are indicated in black, and *trans*-acting factors as hatched boxes. The transcription initiation site is shown by the right-angled arrow. (b) Hypothetical transcription initiation complex. Interaction of bound *trans* factors with RNA polymerase II by looping of DNA; protein-protein interactions are suggested by links between the *trans* factors.

steroid and thyroid hormone receptor structure and DNA binding characteristics, and also the identification of the pituitary tissue-specific factor Pit-1/GHF-1; these will be considered in detail below.

## TISSUE-SPECIFIC EXPRESSION

The PRL and GH genes are present in all cells but are expressed only in pituitary or placental tissue; indeed, the respective levels of mRNA for hGH-N and hCS may differ by as much as 10 000-fold despite considerable sequence homology, raising the intriguing question of the possible mechanisms which confer this tissue specificity of gene expression. In the case of both PRL and GH it is noteworthy that segments of the 5'-flanking sequence are markedly conserved between species, suggesting their importance in gene regulation. The PRL and GH genes have normally been considered separately, and they will be discussed separately here, before a picture of our current understanding is summarized. However, the mechanisms responsible for conferring tissue specificity for PRL and GH gene expression may, in fact, be at least partly shared, and a pituitary-specific *trans*-acting factor(s) has recently been identified which appears to regulate tissue-specific expression of the PRL and GH genes and possibly also the TSH- $\beta$  subunit gene (Nelson et al, 1988); this factor, Pit-1 or GHF-1, has subsequently been cloned and identified by two independent groups (Bodner et al, 1988; Ingraham et al, 1988). Whether this factor in fact regulates both PRL and GH has been disputed (Castrillo et al, 1989) but evidence that some similar tissue-specific targeting mechanism operates *in vivo* is provided



by the finding that upstream elements of the GH gene can direct expression of the gene in transgenic mice to lactotrophs and thyrotrophs as well as to somatotrophs (Lira et al, 1988).

## **Prolactin**

### *Cis elements*

The technique of transfection analysis has been used to study the role of the 5'-flanking DNA sequences of the rat PRL gene. This technique involves the construction of fusion genes which contain putative regulatory elements linked to reporter genes, such as bacterial chloramphenicol acetyl transferase (CAT); the expression of such a reporter gene's product can be readily measured in mammalian cell extracts to indicate the level of promoter activity. By transfecting rat pituitary GH<sub>3</sub> cells with a series of PRL 5'-flanking DNA-CAT constructs and monitoring the transient expression level of CAT, Nelson et al (1986) identified two regulatory sequences whose deletion led to a drop in CAT gene transcription. A distal element located between -1831 and -1530 base pairs (bp) upstream of the transcription initiation site (or 'cap site') accounted for 98-99% of basal tissue-specific expression, and a proximal element (-422 to -36 bp) accounted for 1-2% of activity. Both *cis* elements acted as enhancers; that is, they conferred cell-type specific expression to a 'neutral' viral promoter sequence independent of their position or orientation. Similar transfection studies have demonstrated that 250 bp of the bovine PRL 5'-flanking sequence confer tissue-specific gene expression (Camper et al, 1985).

The functional importance of the proximal region of the PRL 5'-flanking DNA has further been indicated by *in vitro* transcription experiments using nuclear protein extracts to reconstitute transcription from a gene promoter element in a cell-free system. *In vitro* transcription experiments in GH<sub>3</sub> rat pituitary tumour cell extracts suggested that only the proximal regulatory elements were necessary for tissue-specific expression of the rPRL gene: accurate initiation of transcription could be found using 420 bp of 5'-flanking rat PRL DNA, and whereas nuclear proteins from various cell types could direct transcription from viral gene promoters, only pituitary extracts could do so using the PRL promoter template DNA (Cao et al, 1987; Gutierrez-Hartmann et al, 1987; Cao et al, 1988). A pituitary-specific transcription factor binding to an upstream promoter element appears to selectively promote transcription initiation at the +1 site in preference to initiation at a second site at -27 bp upstream which may occur with non-pituitary extracts (Barron et al, 1989).

### *Cis/trans interactions*

The first demonstration of nuclear proteins binding to 5'-flanking segments of the rPRL gene suggested that they could bind to two long stretches of DNA extending as far as -4800 bp upstream of the cap site (White et al, 1985). However, two methods have been increasingly used to locate much





more precisely the nucleotide sequences which bind *trans*-acting tissue-specific proteins. They both rely on the fact that protein binding protects DNA from non-sequence-specific nuclease digestion, either cutting from the ends with exonuclease III, or cutting within fragments with deoxyribonuclease (DNase I).

*Proximal protein binding sites.* Different studies and different techniques have yielded slightly differing results but up to four protein binding sites have been identified within the first 210 bp 5' to the cap site, as shown in Figure 3 (Elsholtz et al, 1986; Cao et al, 1987; Gutierrez-Hartmann et al, 1987; Nelson et al, 1988; Schuster et al, 1988). At least three of these proximal sites are pituitary specific, as the protein binding is seen only with pituitary cell extracts and not with proteins from other tissues.

Of the four sites, the most proximal, between positions -30 and -78, has also been found to behave as an enhancer element which confers the transcriptional response to epidermal growth factor (EGF) and phorbol ester (Elsholtz et al, 1986). Using 'gel retardation' assays it has been found that the electrophoretic mobility of this DNA element is reduced by protein binding, specifically with extracts from pituitary cells but not from other cell types (Elsholtz et al, 1986; Schuster et al, 1988), again providing independent evidence for tissue-specific nuclear protein interactions with this region. The second site appears in some studies not to be tissue-specific (Gutierrez-Hartmann, et al, 1987). However, the fourth of these four proximal sites corresponds closely to a sequence identified by Lufkin and Bancroft (1987) which is able to direct gene transcription in hybrid pituitary-fibroblast fusion cells but not in fibroblast cells alone; these data provide direct evidence for the presence in pituitary cells of a diffusible protein factor that can increase transcriptional rate, but also show that the protein binding to this sequence is necessary for gene expression.

*Distal binding sites.* Using DNase I footprinting, Nelson et al (1988) found that, in addition to the four proximal protein binding sites described above, there are four distal sites between positions -1579 and -1718 in the rat PRL gene. None of the total of eight sites were found with non-pituitary protein preparations but, interestingly, the sites mutually competed for protein binding, suggesting that all eight bound a single protein factor; in addition, protein binding was effectively reduced by competition with the tissue-specific enhancer sequences of the rat GH gene. Kim et al (1988) demonstrated only one of these four sites which was distinct from the oestrogen response element (ERE), and showed that mutations in this site abolished nuclear protein binding and reduced the transcriptional enhancer activity of the overall distal region several-fold.

The data of Nelson et al (1988) suggested the existence of a single pituitary-specific factor named Pit-1, which binds to the enhancer regions of both the PRL and GH genes. This factor may be the same as the GH-specific factor GHF-1 described by Lefevre et al (1987); the cDNA coding for Pit-1/GHF-1 has now been cloned independently by the two groups (Bodner et al, 1988; Ingraham et al, 1988), though it has been disputed

whether this protein in fact binds to the PRL promoter (Castrillo et al, 1989).

### *Human prolactin gene*

The tissue-specific regulation of the human prolactin gene has only been much more recently studied but the organization of at least the proximal element of the 5'-flanking region appears similar. In vitro transcription studies have shown that 740 bp of hPRL promoter DNA can direct tissue-specific expression of the CAT reporter gene with pituitary cell but not HeLa cell extracts. DNase I footprinting studies have indicated pituitary-specific *trans* factor binding to three sites, corresponding to the highly homologous sites 1, 3 and 4 in the rat gene, except that 'site 4' is slightly further upstream (Lemaigre et al, 1989a); (Figure 3).

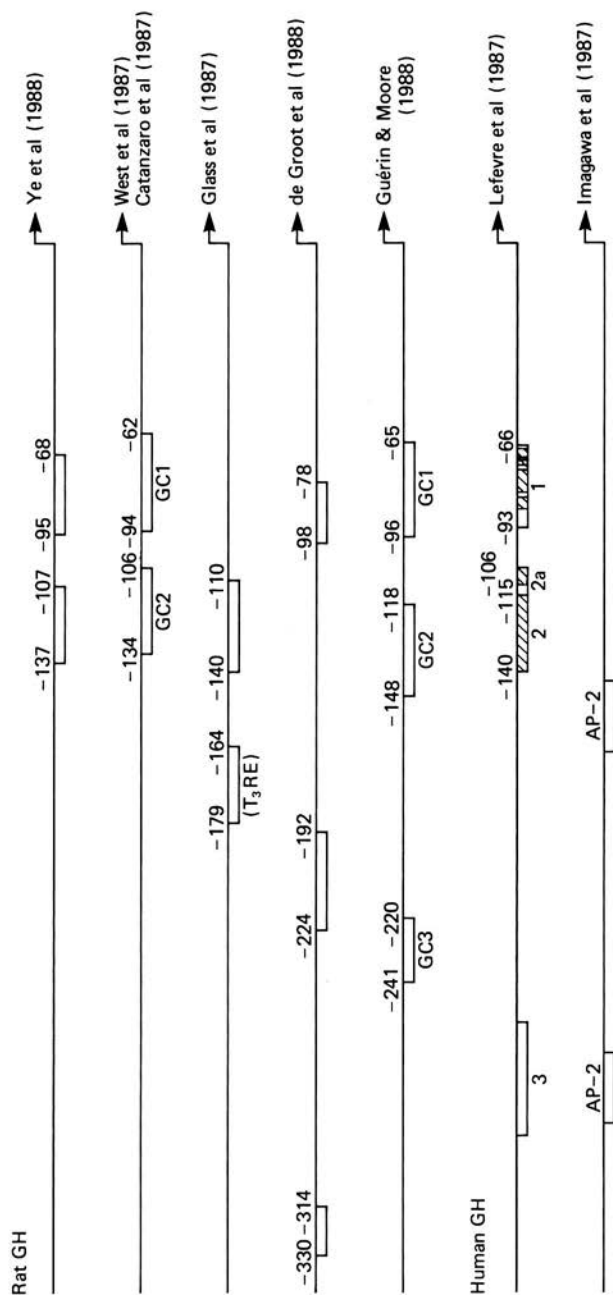
## **Growth hormone**

### *Cis elements*

Transfection studies using rat GH promoter-CAT fusion genes have shown that as little as 235 bp of rat GH 5'-flanking sequence are able to confer cell-type specificity (Nelson et al, 1986). The degree of expression fell dramatically with deletion of more than 181 bp 5' to the cap site, suggesting the presence of a tissue-specific enhancer element between -181 and -235 bp; however, neither elements extending from -235 to -146, nor from -181 to the cap site, were fully effective, suggesting that the putative enhancer element was relatively extensive. Independent transfection studies using much longer stretches of rat GH promoter and 5'-flanking sequences (1800 bp) confirmed that CAT expression was restricted to pituitary cells, but this restriction of specificity was lost when 5' sequences were deleted down to the -309 or -183 positions (Larsen et al, 1986a). This suggested the presence of upstream repressor elements which normally prevented gene activation in non-pituitary cell types. Although these data conflict with those of Nelson et al (1986) in suggesting that tissue specificity is not conferred solely by 235 bp of 5'-flanking sequences, further data from Larsen et al (1986b) showed that sequences between -183 and -202 were able to confer thyroid hormone responsiveness, but only in pituitary-derived cell lines. Thus, there is evidence for overlap between these two aspects of gene regulation: a thyroid hormone response element (discussed in more detail below and elsewhere) may respond in a tissue-specific manner; or, conversely, hormone responses may be mediated by interaction with tissue-specific *trans* factor binding, and a larger enhancer region consisting of a thyroid hormone response element may be functionally linked with tissue-specific elements (Ye et al, 1988).

### *Cis/trans interactions*

Gel retardation analysis has been used to demonstrate tissue-specific protein interactions with rat GH 5'-flanking sequences by Ye and Samuels (1987).



Nuclear protein extracts from pituitary GC or GH4C1 cells selectively retarded the electrophoretic migration of  $-104/+7$  and  $-236/-146$  restriction fragments, whereas nuclear proteins from rat hepatoma or fibroblastic cells failed to do so.

More precise localization of protein-binding sites within rat GH 5'-flanking DNA has been undertaken with DNase I footprinting studies. As for the studies on the rPRL gene, the exact details vary slightly between different reports, but two consensus footprints may be deduced between positions  $-98$  and  $-62$  ('GC1'), and  $-140$  and  $-106$  ('GC2'), as summarized in Figure 4 (Catanzaro et al, 1987; Glass et al, 1987; de Groot et al, 1988; West et al, 1987; Ye et al, 1988). Other footprints have been shown further upstream (Glass et al, 1987; de Groot et al, 1988); a site at  $-220/-241$ , termed 'GC3' has recently been shown to be pituitary specific but binds a different protein factor from the GC1 and GC2 sites (Guérin and Moore, 1988). The spatial relationship between the two proximal elements is important, and Ye et al (1988) showed that proteins must bind on the same side of the DNA helix to preserve full efficacy of this region, suggesting that contact between two DNA bound proteins.

### *Human GH gene*

Transfection studies of the human GH gene showed that 500 bp of 5'-flanking DNA allowed expression of a reporter gene in rat GC or GH<sub>3</sub> cells but not in the non-pituitary Rat-1, HeLa or placental JEG-4 cell lines (Lefevre et al, 1987), suggesting the presence of a cell-type specific enhancer element within this region. Likewise Cattini et al (1986a), using deletion analysis, found that an element between  $-230$  and  $-180$  was necessary for pituitary-specific expression.

Using DNase I footprinting, Lefevre et al (1987) found three hGH sites binding proteins from rat GC cell extracts at  $-66/-93$  (site 1),  $-106/-140$  (site 2) and  $-254/-290$  (site 3); of these three sites, part of site 2 and all of the distal site 3 were also protected by HeLa cell extracts. Thus, two pituitary-specific protein binding sites were identified (sites 1 and 2a) (Figure 4), and they mutually competed for binding of a single protein factor which was named GHF-1. Mutations within either site abolished GHF-1 binding. In fact both GHF-1 sites contain regions very highly homologous to the GC1 and GC2 sites described for the rGH gene promoter (Catanzaro et al, 1987; West et al, 1987) and have similar locations. GHF-1 is not the only protein involved, however, and Imagawa et al (1987) have demonstrated footprints from binding of purified activator protein-2 (AP-2) to two further sites in the hGH gene 5'-flanking region: AP-2 is a *trans* acting factor which is involved in intracellular messenger signalling; it is not a tissue-specific factor, and, indeed, the more distal site in fact coincides with the non-tissue-specific site 3 found by Lefevre et al (1987), as shown in Figure 4. Moreover, Lemaigre et al (1989b) showed by footprinting in the presence of competing oligonucleotides that the non-pituitary-specific factor SP1 binds to part of site 2, and upstream stimulatory factor (USF) binds to the proximal part of site 3 (the distal part binding AP-2).

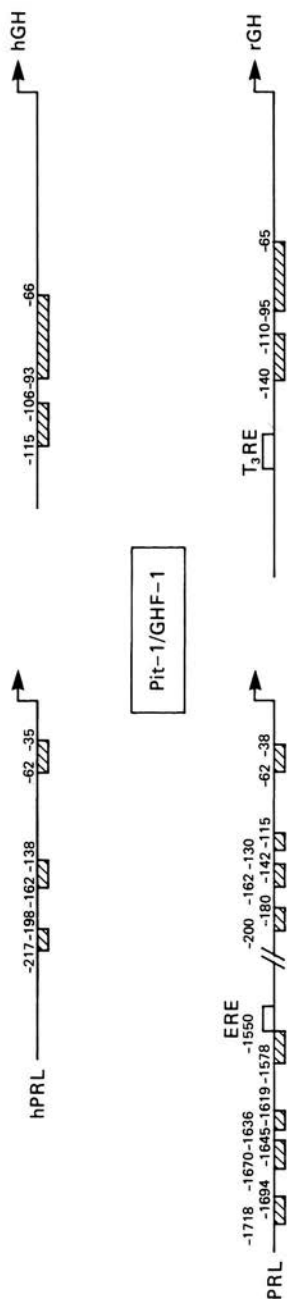
The functional importance of these protein-binding regions in maintaining hGH gene transcription has been demonstrated by *in vitro* transcription experiments. Bodner and Karin (1987) found that 289 bp of hGH 5'-flanking DNA were able to support transcription with pituitary GC cell nuclear protein extracts efficiently. HeLa cell extracts alone were much less efficient in supporting transcription from the hGH promoter template but complementation with pituitary extracts increased transcriptional activity. Mutations in each of the two GHF-1 binding sites caused a severe reduction in transcriptional activity of the template.

## Summary

For both the PRL and GH genes, in both man and rat, there is evidence for the existence of DNA sequences which are responsible for tissue-specific gene expression. Binding of nuclear proteins to these regions has been demonstrated, and appears to be responsible for establishing basal levels of gene transcription. Two sites within the rat and human GH promoter DNA are homologous and appear to bind the same protein factor (GHF-1, or GC1/GC2); eight sites upstream of the rPRL gene, and at least three sites for the hPRL gene, also appear to bind a single *trans*-acting factor (Pit-1) and sequences of the GH gene compete for this protein binding.

Thus it appears that a single protein in both rat and human (Pit-1/GHF-1) may be responsible for directing expression of both genes (Figure 5) and may also bind to the promoter of the TSH- $\beta$  gene (Nelson et al, 1988). The cDNA encoding Pit-1/GHF-1 has recently been cloned (Bodner et al, 1988; Ingraham et al, 1988). However the two reports disagree on the crucial point as to whether Pit-1/GHF-1 protein can bind to the rPRL gene. Castrillo et al (1989) have recently purified GHF-1 from GC pituitary cells and identified it as a 33kD polypeptide. This material bound to two sites on rGH 5'-flanking DNA, but not to rPRL sequences, whereas a separate extracted protein bound to five sites (including the four previously recognized sites) on the rPRL proximal upstream enhancer element. Expression of the gene for Pit-1/GHF-1 at high levels in transiently transfected heterologous HeLa cells selectively activates expression of fusion genes containing either the rPRL or the rGH promoter sequences (Ingraham et al, 1988), but it still remains to be clearly established whether expression of the rPRL gene *in vivo* can be directed by Pit-1/GHF-1 or requires a separate yet uncharacterized *trans*-acting factor.

Pit-1/GHF-1 contains a DNA-binding region closely similar to the homeodomain first described in a class of proteins involved in embryonic development in *Drosophila*. However, unlike many other transcriptional regulators, Pit-1/GHF-1 is expressed exclusively in the pituitary gland, in lactotrophs and somatotrophs and also a subset of thyrotrophs. The reasons for the restriction of Pit-1/GHF-1 expression to the pituitary have yet to be clarified; however GH gene expression is extinguished when pituitary cells are fused with heterologous somatic L-cells, and this process is accompanied by loss of GHF-1 mRNA accumulation and protein production (McCormick et al, 1988). This suggests the existence of a repressor that is



**Figure 5.** Comparison of protein binding sites in the human and rat PRL and GH genes for pituitary-specific factor(s). Proposed binding sites for Pit-1 and/or GHF-1 are shown as hatched boxes, and oestrogen response element (ERE) and thyroid hormone response element (T<sub>3</sub>RE) are shown as open boxes.

involved in the tissue-specific expression of Pit-1/GHF-1 itself.

The most direct demonstration that *cis*-active elements binding Pit-1/GHF-1 are indeed important *in vivo* in directing tissue-specific expression has come recently from experiments using transgenic mice. Lira et al (1988) showed that upstream sequences of the rat GH gene were able to direct expression of a linked human GH structural gene predominantly in pituitary somatotrophic cells, but also in lactotrophs and thyrotrophs, while not in gonadotrophs or corticotrophs. Thus a working hypothesis suggests that a single *trans*-acting factor directs cell-type-specific expression of these genes to three cell types within the pituitary. The final level of gene expression within a single cell type presumably reflects the integration of a variety of other signals such as thyroid hormone, glucocorticoids, sex steroids and peptide hormones. The proximity of Pit-1/GHF-1 sites to hormonally responsive regions in both genes is noteworthy, and it may be that some hormonal signals exert their effect by modifying the binding of tissue-specific factors, or by covalent modification of *trans* factors that are already bound to DNA sequences, increasing or decreasing their ability to interact with the RNA polymerase II transcription initiation complex.

## HORMONAL AND INTRACELLULAR REGULATION

The cell-type-specific characteristics of PRL and GH gene regulation appear to be very similar, and the mechanisms may indeed be partly mediated by a common factor for both genes. However, within the pituitary their hormonal regulation differs markedly. Indeed if cell-type-specific expression by Pit-1/GHF-1 targets the GH gene to lactotrophs and thyrotrophs as well as somatotrophs, additional restrictive or permissive cell-type specification mechanisms must exist to explain the actual targeting of endogenous gene expression to a single cell type. A large number of hormonal signals may act upon given cell types, and in principle these might act either independently or by modulating tissue-specific control mechanisms. Thus, hormonally induced *trans*-acting factor activation may either have a direct effect on 'dedicated' hormone-responsive DNA sequences, or an indirect effect by modulating tissue-specific protein (for example Pit-1/GHF-1) interactions with DNA. The latter possibility has recently been proposed to explain the action of the glucocorticoid receptor on the PRL gene (Adler et al, 1988).

Oestrogen and thyroid hormones exert their effects by binding to specific nuclear receptors which have been shown to be DNA-binding proteins, and much of our present understanding of receptor-DNA interactions derives from exciting recent work on nuclear receptor structure (reviewed by Evans, 1988, and elsewhere in this volume). But peptide hormones and dopamine, which bind to membrane receptors, must act through intermediate intracellular signalling systems via 'second messengers' (Berridge, 1985; B. L. Brown et al, 1985), and the ways in which these act to regulate gene transcription is much less clear. For these substances, the relevant pathways linking second messenger formation to *trans*-activating proteins have yet to be characterized: do the various intracellular signals linked to given mem-



brane receptors initiate a cascade which converges on to a final common pathway in the nucleus, or is the variety of second messenger signals an important channelling mechanism allowing integrated effects on a gene of a wide variety of different *trans*-activation proteins?

## Prolactin

### *Nuclear hormone receptors*

PRL gene transcription is stimulated or inhibited by a variety of hormones (Table 1); for some of these, hormone-responsive elements have been identified in the 5'-flanking region of the rPRL gene, but no studies have yet characterized the regulation of the hPRL gene.

**Table 1.** Summary of known effects of hormones and intracellular messenger substances on gene transcription or mRNA accumulation.

	rPRL	rGH	hGH
<i>Hormones</i>			
Oestrogen	+		
Thyroid hormone	-	+	-
Glucocorticoid	-	+	+
Vitamin D	+		
Thyrotrophin-releasing hormone	+	-	
Growth hormone releasing factor		+	+
Dopamine	-		
Somatostatin		?-	
Vasoactive intestinal peptide	+		
$\gamma$ -Aminobutyric acid	-		
Insulin	+	+/-	
Insulin-like growth factor I		-	-
Epidermal growth factor	+		
<i>Intracellular signals</i>			
Cyclic AMP	+	+	+
Calcium	+	+	
Phorbol ester	+		

rPRL, rat prolactin; rGH, rat growth hormone; hGH, human growth hormone; +, stimulation; -, inhibition.

**Oestrogen.** Physiological effects of oestrogen on PRL mRNA accumulation *in vivo* have been clearly shown by *in situ* hybridization (Steel et al, 1988). *In vitro*, 17- $\beta$ -oestradiol causes increased accumulation of PRL mRNA in rat pituitary cells (Stone et al, 1977) and this effect is due to a rapid stimulation of the rate of gene transcription (Maurer, 1982a; Shull and Gorski, 1984; Waterman et al, 1988). The stimulatory effect of oestrogen is exerted by direct binding of oestrogen receptor (ER) to DNA (Green and Chambon, 1986; Evans, 1988) and an oestrogen response element (ERE) in rat PRL 5'-flanking DNA has been located more than 1500 bp distant from the transcription initiation site (Maurer, 1985; Maurer and Notides, 1987; Waterman et al, 1988; Somasekhar and Gorski, 1988). It is interesting to

note, however, that this ERE is close to a region of DNase I hypersensitivity, which may be important in the mechanism of transcriptional activation (Durrin and Gorski, 1984; Durrin et al, 1984); but the ERE is also immediately adjacent to one of the four distal Pit-1 binding sites described by Nelson et al (1988), suggesting possible interactions between oestrogen receptor and a separate tissue specific protein (see also Kim et al, 1988).

**Glucocorticoids.** Dexamethasone inhibits bovine PRL gene transcription by an action exerted within the first 250 bp of 5'-flanking DNA (Camper et al, 1985). More recently it has been shown that dexamethasone has a similar effect on proximal promoter elements of the rPRL gene (Adler et al, 1988; Somasekhar and Gorski, 1988). However, interestingly, it was found that an inhibitory effect of oestrogen on the proximal region of the rPRL promoter could be expressed in specially manipulated circumstances (by inducing over-expression of the ER); it was then shown that the inhibitory effect of the glucocorticoid receptor (GR) and the over-expressed ER was not exerted by their DNA-binding domain (Adler et al, 1988). Thus, the stimulatory and inhibitory effects of these receptors were exerted by different parts of the molecule, and the negative effects did not involve direct protein-DNA interaction but most probably indirect effects on other DNA-binding proteins. Various protein-protein interactions have recently been proposed for glucocorticoid receptor action on other genes. Glucocorticoid response elements are frequently clustered with other *trans* factor binding sites (Schüle et al, 1988) and their spatial arrangement is critical for hormonal regulation. Indeed in the pituitary glycoprotein  $\alpha$  subunit gene, Akerblom et al (1988) found overlap between the cAMP responsive region of the gene promoter and the GR binding element, and suggested that negative regulation could result from interference of a hormone receptor-DNA complex with other positive *trans*-activating proteins.

**Thyroid hormone and vitamin D.** Two other nuclear receptors, binding  $T_3$  and vitamin D respectively, are closely related (Evans, 1988) and seem likely to exert effects on the PRL gene but, in contrast to the rapid advances in knowledge of GH gene regulation by  $T_3$ , much less is known about their mechanism of action on the PRL gene. It has been shown that  $T_3$  causes inhibition of mRNA accumulation in rat pituitary cells (Maurer, 1982b; Stanley and Samuels, 1984), and that the weak thyroid hormone agonist drug phenytoin has similar effects (Davis et al, 1986). Vitamin D has been shown to stimulate cytoplasmic accumulation of PRL mRNA (Wark and Tashjian, 1983) and this stimulation is antagonized by glucocorticoids (Wark and Gurtler, 1986). Transient transfection of GH<sub>4</sub> cells with plasmid expression vectors containing the *c-erb-A*  $\alpha$  and  $\beta$  genes (encoding  $T_3$  receptors) have shown  $T_3$ -inducible inhibition of expression of a co-transfected PRL promoter-reporter gene construct, providing the first evidence that *c-erb-A* proto-oncogenes can directly affect the PRL (and also GH) gene expression (Forman et al, 1988). It seems reasonable to speculate that nuclear receptors for  $T_3$  and vitamin D may interact with DNA or other *trans* factors in a manner similar to the ER and GR, and indeed it has been

found that  $T_3$  receptor may bind to oestrogen response elements in a transcriptionally inactive form, to decrease the overall level of gene transcription (Glass et al, 1988).

### *Peptide and dopamine regulation*

Thyrotrophin-releasing hormone (TRH) stimulates rPRL gene transcription within minutes, and increases cytoplasmic mRNA accumulation (Laverriere et al, 1983; Murdoch et al, 1983; White and Bancroft, 1983). In addition, TRH increases the half-life of cytoplasmic mRNA from 17 to 27 h (Laverriere et al, 1983). Similar stimulation is seen with EGF (White and Bancroft, 1983; Murdoch et al, 1985b). Supowit et al (1984) showed by transfection studies that 3000 bp of 5'-flanking DNA conferred EGF responsiveness on to a reporter gene and an EGF-responsive element was located within the first 78 bp by Elsholtz et al (1986). For the bovine PRL gene also, Camper et al (1985) have shown that 250 bp were sufficient to allow stimulation by TRH and EGF, implying that this region of DNA contains the necessary sequences for recognition of the intracellular signals generated by these two peptide hormones. Vasoactive intestinal peptide (VIP) also elevates PRL mRNA levels in pituitary cells (Carrillo et al, 1985). Dopamine inhibits PRL gene transcription, as does the dopaminergic drug bromocriptine (Maurer, 1980, 1981, 1982c). Finally,  $\gamma$ -aminobutyric acid (GABA) has an inhibitory effect on PRL mRNA accumulation (Loeffler et al, 1985), while insulin is stimulatory (Prager et al, 1988).

These various hormones are thought to act through different intracellular mechanisms: TRH stimulates the hydrolysis of membrane phospholipids resulting in protein kinase C activation and calcium mobilization, and leading to phosphorylation of cytoplasmic and nuclear proteins (Drust and Martin, 1982; Murdoch et al, 1982; Gershengorn, 1986); EGF activates a transmembrane protein kinase (Stoschek and King, 1986); VIP activates adenylate cyclase (Gourdji et al, 1979), which results in phosphorylation of a different group of proteins than with TRH action (Drust et al, 1982; Murdoch et al, 1983). Thus the rPRL gene is likely to be controlled by a number of interacting intracellular messenger systems (Bancroft et al, 1985; Murdoch et al, 1985a).

### *Intracellular messenger pathways*

**Cyclic AMP.** Cyclic AMP has been shown to increase rPRL gene transcription (Maurer, 1981), and forskolin (which activates adenylate cyclase) increases cytoplasmic mRNA accumulation (Murdoch et al, 1982b; Dave et al, 1987). Two cyclic AMP responsive elements (CREs) (reviewed by Roesler et al, 1988) have been defined: one of these CREs has been found in various genes including the pituitary glycoprotein  $\alpha$ -subunit (Deleage et al, 1987), VIP (Tsukada et al, 1987) and somatostatin (Montminy and Bilezikjian, 1987). The corresponding *trans* factor is the protein CREB, whose DNA binding and transcriptional efficacy are regulated by phosphorylation (Yamamoto et al, 1988). A second system of cyclic AMP

regulation involves AP-2, which has two binding sites in the hGH promoter (Imagawa et al, 1987) and a potential binding site in the rPRL promoter.

**Calcium.** Two intracellular signals are known to mediate TRH action, namely intracellular calcium and protein kinase C. Intracellular calcium appears to be crucial for PRL gene expression: GH<sub>3</sub> cells grown in low-calcium medium respond to addition of calcium by a dramatic rise in both PRL synthesis and mRNA accumulation (White et al, 1981), which occurs within 10 h. On the other hand, removal of extracellular calcium by EGTA rapidly reduces PRL mRNA to undetectable levels, whereas GH mRNA levels show a smaller, slower fall (Gick and Bancroft, 1985). Calcium ionophores and calcium channel agonists can stimulate PRL gene transcription rate (Hinkle et al, 1988; Laverriere et al, 1988), again suggesting an effect of intracellular calcium concentration *per se*. A requirement for calcium has further been demonstrated by studies using calcium channel blocking agents such as verapamil or nifedipine, and calcium antagonists such as cobalt (Murdoch et al, 1985b; Davis et al, 1988a; Hinkle et al, 1988). Studies with calmodulin antagonist drugs have suggested that PRL mRNA accumulation is calmodulin dependent (Bancroft et al, 1985; Murdoch et al, 1985b; White, 1985). The possible mechanisms for calcium/calmodulin action in the induction of PRL gene transcription may involve the calcium/calmodulin-dependent nuclear protein, DNA topoisomerase, which has DNA-unknottting and relaxing activity *in vitro* (White and Preston, 1988). DNA topoisomerase interacted with three sites within 3000 bases of PRL 5'-flanking DNA, and one of these sites was within the first 100 bp (White and Preston, 1988). Further recent evidence from transfection studies has suggested that a 'calcium regulatory element' may lie within the first 174 bases of the 5'-flanking DNA (Jackson and Bancroft, 1988).

**Protein kinase C.** PRL gene transcription is stimulated by activation of protein kinase C with phorbol esters (Murdoch et al, 1985b). Studies of rPRL gene 5'-flanking DNA have demonstrated an upstream phorbol ester responsive element (Supowit et al, 1984). This in fact coincides exactly with the EGF-responsive element, and lies between -78 and -35 bases upstream from the transcription start site (Elsholtz et al, 1986). This region of DNA is clearly of major interest as it forms part of a putative calcium-regulatory element and a site of DNA topoisomerase action (Jackson and Bancroft, 1988; White and Preston, 1988), but also represents the first proximal binding site of the tissue-specific factor Pit-1/GHF-1 (Nelson et al, 1988). The mechanisms of protein kinase C action on gene transcription may involve allosteric modifications of proteins already bound to DNA, as nuclear proteins from phorbol ester-stimulated cells behaved no differently in their DNA binding from those obtained from unstimulated cells (Elsholtz et al, 1986).

In summary, responses to various intracellular messengers seem to involve proximal 5'-flanking sequences in the rPRL gene, and the relevant response elements may coincide with binding sites for a tissue-specific protein factor. However the distal enhancer region may also be involved,

and Day and Maurer (1989) have recently shown that a distal element (-1713/-1495) conferred regulation by TRH, EGF and cyclic AMP onto a reporter gene. This regulation was equally effective as that seen with the proximal enhancer element, and a synergistic interaction was found between these agents and oestradiol, suggesting co-operative interactions between protein-binding *cis*-acting elements in transcriptional control.

### Growth hormone

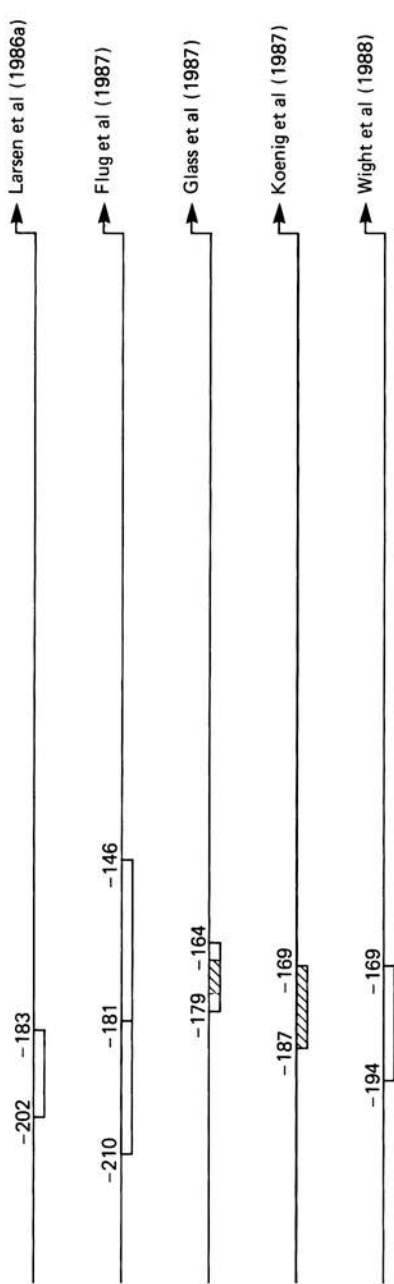
The number of probable hormonal regulators of the GH gene is rather smaller than for PRL (Table 1). The best studied of these regulators is thyroid hormone (reviewed by Samuels et al, 1988), which is well known to be a major regulator of GH secretion *in vivo*, at least in the rat; the peptide hormones, growth hormone releasing factor (GRF), somatostatin and IGF-I have so far received much less attention.

#### *Thyroid hormone*

Rat GH gene transcription is induced by  $T_3$  by up to tenfold *in vitro* in the pituitary GC cell line (Spindler et al, 1982; Nyborg et al, 1984; Yaffe and Samuels, 1984). This induction coincides with  $T_3$  receptor occupancy and alterations in chromatin structure (Nyborg and Spindler, 1986) and requires the presence of yet uncharacterized short-lived proteins (Santos et al, 1987). The *c-erb-A* proto-oncogene, which encodes the  $T_3$  nuclear receptor has now been shown in co-transfection experiments to induce GH gene expression (Forman et al, 1988).

The *cis* elements involved in this  $T_3$  regulation have been more precisely located by transfection studies using various lengths of rat GH gene promoter linked to reporter genes. In a series of experiments with cells that had stably integrated the exogenous constructs into their genome,  $T_3$  induction could be observed with progressively smaller promoter fragments, from 1800 bp (Casanova et al, 1985) down to 235 bp of 5'-flanking DNA (Crew and Spindler, 1986). Further analysis showed the simultaneous presence of a positive and a negative thyroid response element (Wight et al, 1987); the first of these behaved as an enhancer (at position -254/-241) but the second did not, and overlapped with the TATA box (position -46/-21). Later studies with staged deletions of 5' and 3' regions in rGH fragments (from which the negative element had been deleted) mapped the borders of the positive element to position -194/-169 (Wight et al, 1988) corresponding closely to a  $T_3$  receptor footprint described by Koenig et al (1987), and which contains part of a DNA sequence that binds the *c-erb-A* proto-oncogene translation product (Glass et al, 1987) (Figure 6.)

In another series of studies (Larsen et al, 1986a; Flug et al, 1987) the transcription of the CAT reporter gene was measured in transient expression 2 to 3 days after cell transfection. The data obtained are slightly different from the results described above (Figure 6), perhaps because the constructs are not integrated in the host cell genome while  $T_3$  has an important effect on GH cell proliferation, or possibly because the higher



**Figure 6.** Summary of proposed positive T<sub>3</sub> response elements in the rat GH gene. Cross-hatched elements are protein binding sites determined by footprinting analysis, and open boxes are based on results of transfection experiments.



copy number of constructs per cell might compete for binding of some regulatory factors. The apparent  $T_3$  response element is close to the *cis* elements shown to bind nuclear proteins (including  $T_3$  receptor) and to be necessary for tissue-specific expression. Larsen et al (1986a) found that sequences between positions -183 and -202 were able to confer thyroid hormone responsiveness, but only in pituitary cell types, indicating that a thyroid hormone response element itself may have tissue-specific characteristics. Ye et al (1988) proposed that two interacting tissue-specific *cis* elements (at -137/-107 and -95/-65) are functionally linked with the  $T_3$  response element to act as an enhancer-like unit which confers both cell-type-specific and  $T_3$ -regulated gene expression. Both interacted to confer regulated expression to heterologous promoters, and it was suggested that the  $T_3$ -receptor-DNA complex is able to enhance the function of the cell-specific elements to increase the level of rat GH gene expression. Finally, gel retardation and DNase I footprinting studies have shown that partially purified  $T_3$ -receptor preparations show binding to upstream regions of the GH gene, and that there may in fact be multiple sites which can interact with receptor within the region -530/+7 (Ye and Samuels, 1987; Aprelletti et al, 1988; de Groot et al, 1988; Lavin et al, 1988).

### *Glucocorticoids*

Transcription of the endogenous rat GH gene is induced by five to twenty-fold in the GH cell line (Martial et al, 1977; Evans et al, 1982; Wegnez et al, 1982). Initial attempts at studying the mechanism of glucocorticoid action were undertaken using heterologous (non-pituitary) recipient cells which had stably integrated the transfected rGH gene into their genome. This experimental approach led to the production of mRNAs that had aberrant transcription initiation sites, but whose amounts were nevertheless variably glucocorticoid inducible (Doehmer et al, 1982; Kushner et al, 1982; Karin et al, 1984; Miller et al, 1984). An rGH promoter region as short as 248 bp was able to confer glucocorticoid induction to a reporter gene in heterologous cells (Heiser and Eckhardt, 1985), but in transient transfection analyses in pituitary cells using rGH 5'-flanking DNA linked to a reporter gene, Crew and Spindler (1986) and Flug et al (1987) found only minimal glucocorticoid effects. Birnbaum and Baxter (1986) found that a promoterless rGH gene was glucocorticoid inducible, with mRNA of a nearly correct size, suggesting that glucocorticoid regulatory elements were contained downstream, not upstream, from the transcription initiation site. A glucocorticoid response element has been described in the first intron of the human GH gene (Slater et al, 1985), and although such a site has not yet been shown in the rat GH gene, its first intron does contain a TGTCT sequence typical of GR binding sites.

### *Peptide hormone regulation and intracellular messenger systems*

Hypothalamic GRF stimulates rat GH gene transcription in primary cell cultures (Barinaga et al, 1983, 1985; Gick et al, 1984) and also in transgenic

mice over-expressing the human GRF precursor gene (Hammer et al, 1985a). Somatostatin reduces rat GH mRNA accumulation (Wood et al, 1987), although a separate study failed to show short-term effects on transcriptional rate (Barinaga et al, 1985). Insulin blocks  $T_3$  stimulation of rGH secretion (Melled and Slanina, 1985) and may inhibit GH mRNA accumulation (Melled et al, 1985a; Yamashita and Melmed, 1986), but, depending on culture conditions, other workers have found that insulin may also exert stimulatory effects (Isaacs et al, 1987a). Ectopic hypersecretion of GH by transplanted tumours in the rat has been shown to inhibit GH mRNA accumulation in vivo (Yamashita et al, 1986a), and this autoregulation probably involves negative feedback from insulin-like growth factor I (IGF-I) (Yamashita and Melmed, 1987).

Transcription of the rat GH gene may be modulated independently of GH release: GRF stimulation of GH release is highly dependent upon extracellular calcium whereas both basal and GRF-stimulated GH gene transcription are much less so (Barinaga et al, 1985). Rat GH mRNA accumulation is somewhat inhibited by removal of extracellular calcium but this effect is much less marked than for PRL mRNA (Bancroft et al, 1985; Gick and Bancroft, 1985). GRF is thought to exert its effect on somatotrophs through stimulation of adenylate cyclase, and its effects on rat GH gene transcription may be mimicked by exogenous cAMP (Clayton et al, 1986) and adenylate cyclase activation, but not by activation of protein kinase C or cell depolarization, which stimulate only secretion of GH (Barinaga et al, 1985; Morita et al, 1987).

### *Human GH gene*

Analysis of stable transformants obtained by transfection of the human GH gene with 500 bp of promoter sequences into GH<sub>3</sub> cells (Cattini et al, 1986b) has shown that  $T_3$  inhibits hGH gene transcription while stimulating that of the rat GH gene. However this  $T_3$  inhibition is not clearly linked to the hGH promoter as it could not be shown in transient expression studies using the CAT reporter gene (Cattini and Eberhardt, 1987; Brent et al, 1988). The endogenous gene may not always behave in a similar fashion to a transfected construct. Transcriptional control of the endogenous human GH gene has been the subject of limited study, using tissues obtained from surgically removed pituitary somatotroph adenomas. Isaacs et al (1987b) found stimulation of hGH gene mRNA accumulation by dexamethasone, but no effect of  $T_3$ , in contrast to the inhibitory effects of  $T_3$  seen in 7 of 11 rat GC cell lines stably transfected with a human GH gene construct. These differences between transfected and endogenous genes, and the variability of the transfection data, suggest that the cellular environment is critically important in determining the ultimate response to a hormonal stimulus.

Although a  $T_3$  response element has not yet been precisely identified in the human gene,  $T_3$  receptor from lymphoblastic IM-9 cell nuclear extracts is able to bind specifically between positions -129 and -290 in the 5'-flanking region of the gene (Barlow et al, 1986), a position similar to that of the positive  $T_3$  response element in the rat gene. Glucocorticoids stimulate



expression of the transfected human GH gene (Robins et al, 1982; Cattini et al, 1986b; Brent et al, 1988) similar to their effect on the rat gene, and glucocorticoid response element has been demonstrated within the first intron of the gene, that is, downstream of the transcription initiation site (Eliard et al, 1985; Moore et al, 1985; Slater et al, 1985). A weaker glucocorticoid receptor binding site found within the promoter region (Eliard et al, 1985; Slater et al, 1985) could be responsible for the hGH-CAT response to the hormone (Cattini and Eberhardt, 1987).

Little work has been reported on the effects of peptide hormones on the human GH gene, but Yamashita et al (1986b) showed suppression of hGH mRNA levels by IGF-I using cultured cells from five human pituitary adenomas; IGF-I also prevented GRF-induced stimulation of GH mRNA levels. The intracellular messenger systems which may regulate GH gene transcription in the human pituitary are not yet known, but Brent et al (1988) showed that the hGH promoter was cAMP-responsive, and preliminary data have shown stimulation of endogenous hGH mRNA levels and GH release by GRF and by cAMP, whereas stimulation of protein kinase C caused only GH release without affecting mRNA levels (Davis et al, 1988b). These findings are similar to results obtained using normal rat pituitary tissue (Barinaga et al, 1985; Morita et al, 1987), and suggest that in some tumours at least, the intracellular mediators affecting hGH gene regulation are similar to those studied in the normal rat pituitary.

## Summary

For both the PRL and GH genes, a wide variety of hormones and intracellular signalling systems appear to have significant transcriptional effects. While basal expression of the PRL or GH genes requires the interaction of the Pit-1/GHF-1 protein with defined sites in their promoters, modulation of a given level of expression may involve additive effects of adjacent or distant hormone response elements, or modification of Pit-1/GHF-1 or other factors already bound to their *cis* elements.

For the DNA-binding nuclear hormone receptors, the molecular basis of their effects is increasingly well understood, with evidence for both direct protein-DNA effects, and also for indirect protein-protein-DNA interaction. The picture is much less clear for the effects of intracellular second messenger systems; however, a number of interesting models have recently been proposed for cAMP action on other genes, which may be relevant (discussed above and reviewed by Roesler et al, 1988). The cyclic AMP-responsive VIP gene (Tsukada et al, 1987) can be activated in single cells by microinjection of purified catalytic subunit of cAMP-dependent protein kinase (Riabowol et al, 1988) and in a cell-free system the transcription of the urokinase plasminogen activator gene is enhanced by addition either of cAMP itself or of the purified kinase catalytic subunit (Nakagawa et al, 1988). Thus a model may be proposed whereby cAMP (formed as a result of hormone-membrane receptor activation) activates a kinase, resulting in dissociation of its catalytic subunit: this is then translocated to the nucleus and phosphorylates a positive nuclear *trans*-acting factor to enhance gene

transcription by RNA polymerase II. As this group also found that addition of calcium to the cell-free system enhanced in vitro transcription, it is tempting to speculate that such a direct mechanism may mediate the effects of calcium on gene transcription, especially as calmodulin itself may also be translocated into the nuclear matrix (Simmen et al, 1984).

It is now recognized that many proto-oncogene products are directly involved in intracellular signalling pathways and the regulation of gene transcription (Berridge, 1986), and some such proteins are now implicated in the expression of the prolactin and GH genes, apart from the well known example of the T<sub>3</sub> receptor, encoded by the proto-oncogene *c-erb-A* (Sap et al, 1986; Weinberger et al, 1986). It has been shown that GRF, acting via cAMP, can stimulate somatotroph cell division and also rapidly increases levels of *c-fos* mRNA (Billestrup et al, 1986, 1987). The function of the *fos* protein in the pituitary is not yet known, but it may act by modulating the DNA-binding of another proto-oncogene product, *c-jun*, which is now known to encode 'activator protein 1' (AP1), a recognized transcription factor which is responsive to activation of protein kinase C (Lee et al, 1987; Chiu et al, 1988; Curran and Franza, 1988). AP-2, on the other hand, appears to be linked both to protein kinase C and to cAMP (Imagawa et al, 1987), and it will be interesting to learn whether the potential AP-2 binding sites in the prolactin and GH gene promoters are involved in intracellular control, and indeed whether they may interact with Pit-1/GHF-1 binding sites.

In conclusion, evidence is rapidly accumulating for mechanisms of steroid, thyroid hormone and peptide hormone action on gene transcription in a variety of systems. It seems likely that the precise molecular mechanisms for regulated expression of the prolactin and GH genes will be greatly clarified in the near future. The next challenge will be to assess the potential importance of such mechanisms in the pathogenesis of pituitary disease, and this topic will be approached in the next section.

## CLINICAL ASPECTS

Over the past 10 years there has been a rapid advance in knowledge of the structure of the coding and regulatory regions of the PRL and GH genes, and of the normal cellular mechanisms which regulate their expression. While much of the work has been based on rat pituitary tissue, the human genes are increasingly coming under study and it appears that some regulatory mechanisms, just as the gene structures themselves, are strikingly well conserved between species. The clinical situations for which this new knowledge is relevant can be divided into diseases of hormone deficiency (in effect, just GH deficiency) or of hormone excess, namely pituitary tumours secreting PRL or GH.

### Growth hormone deficiency

Several single gene disorders have been found to result in isolated GH deficiency (IGHD) with different modes of inheritance (Phillips et al,

1981a). Type I IGHD is inherited by autosomal recessive transmission, and low but detectable levels of immunoreactive GH are found, but in type IA GH secretion is entirely absent. This latter condition has been found to be due to a large deletion within chromosome 17, which includes the hGH-N gene but not hGH-V (Phillips et al, 1981a, 1981b). As these patients have never been exposed to the authentic pituitary hGH-N protein product, treatment with GH results in a strong initial anabolic response followed by development of hGH antibodies and arrest of growth.

Deletion of the hCS-A gene, which forms part of the GH/CS gene cluster on chromosome 17 has been incidentally found, although this appears to have no adverse effects on the fetus, and is not apparently related to the hGH-N deletion syndrome (Wurzel et al, 1982).

In contrast to IGHD, acquired GH deficiency is relatively common and an important clinical problem but is amenable to GH therapy. Cadaveric GH was formerly used for replacement therapy but recently has been suspected of transmitting viral infection, including Jacob-Creutzfeld disease (P. Brown et al, 1985). Its withdrawal coincided with the development of commercially available recombinant GH following the successful expression of the hGH-N cDNA in bacteria (Goeddel et al, 1979). Commercially available recombinant hGH is identical to authentic hGH, except for a single N-terminal methionine addition (although methionine-free hGH can now be produced in bacteria). Antibody formation in response to met-hGH was due partly to the extra methionine but also partly to contaminating bacterial proteins, and better purification procedures have allowed hGH therapy to become much more readily available than in the past, raising new ethical and economic issues (Grumbach, 1988).

### *Transgenic animals and gene therapy*

The fact that the GH gene could be integrated into the genome of stably transfected heterologous cells, and was expressed and regulated normally, suggested the possibility of introducing the gene into germ cells. Thus Palmiter et al (1982) fused the promoter region of the mouse metallothionein gene to the structural gene encoding rat GH (and later human GH) (Palmiter et al, 1983) and microinjected the fusion gene into fertilized mouse oocytes, which were then placed into the reproductive tracts of foster mothers. Most mice that had stably incorporated the MT-hGH fusion genes into their genome had high serum concentrations of hGH and grew larger than control mice. Synthesis of hGH was enhanced by dietary supplementation with cadmium or zinc, which enhance gene expression from the metallothionein promoter. The use of transgenic manipulations with the GH gene has obvious agricultural implications and the technique has been experimentally extended to produce transgenic rabbits, sheep and pigs (Hammer et al, 1985b).

The expression of *trans*-genes with metallothionein promoters is not tissue specific, but recently *trans*-genes containing GH gene promoters show a remarkable degree of tissue specificity, and Lira et al (1988) have shown that as little as 180 bp of rat GH 5'-flanking DNA are sufficient to direct

hGH gene expression to the pituitary, although it was shown to be produced in lactotrophs and thyrotrophs as well as somatotrophs. These experiments have been considered as models for gene therapy of somatic cells. However, two main technical issues still have to be addressed, namely the precise roles of regulatory DNA elements in controlling and timing tissue-specific expression (Swanson et al, 1985), and the mechanisms for exact targeting of the 'curative' gene to replace its inactive counterpart in recipient cells. One alternative approach may be to consider the implantation of cells which have been stably transfected with the required gene and display regulated hormone expression (Morgan et al, 1987).

### **Pituitary tumours**

Pathological hypersecretion of GH causes the clinical syndromes of gigantism or acromegaly, and generally results from a pituitary somatotroph adenoma. Acromegaly may result from non-pituitary tumours with ectopic GRF production, but ectopic secretion of GH itself may rarely occur, and in one case hGH synthesis by a pancreatic tumour was confirmed by demonstration of mRNA in cytoplasmic extracts (Melmed et al, 1985b). The basic cellular mechanisms which may result in the formation of hormone-secreting pituitary adenomas are not yet understood (Melmed et al, 1986), but a number of recent studies of PRL and GH gene expression have suggested some potential pathological processes which may be relevant. In particular it is interesting to note that for both PRL and GH, hormone secretion and tumour growth appear to be functionally linked, suggesting that the mechanisms for gene regulation elaborated above may have direct relevance for understanding tumour growth.

Abnormalities in membrane transduction proteins linked to intracellular signalling systems have recently been found in pituitary tumours, with evidence for relatively autonomous adenylate cyclase activity in a group of human GH-secreting adenomas (Vallar et al, 1987) and a selective deficiency of  $G_0$  proteins (which may mediate dopamine action) in two dopamine-resistant PRL-secreting rat pituitary tumours (Collu et al, 1988). These abnormalities may well result in abnormal gene expression: investigation of PRL and GH gene regulation in human pituitary tumour tissue is clearly of interest, but until recently such studies have been hampered by lack of large amounts of tissue. Yamashita et al (1986b) showed that hGH mRNA levels were appropriately inhibited by IGF-I and stimulated by GRF in human somatotroph adenomas, as has been found in normal rat pituitary tissue (Barinaga et al, 1983; Yamashita and Melmed, 1987). Another study confirmed this appropriate effect of GRF, but found that somatostatin was ineffective in suppressing GH mRNA levels (Davis et al, 1988b), in contrast to similar studies of normal rat tissue (Wood et al, 1987). Comparable resistance of PRL mRNA accumulation to dopaminergic suppression was also seen in a prolactinoma (Davis et al, 1988b), again suggesting that gene expression was relatively autonomous.

Isaacs et al (1987b) compared the expression of the endogenous hGH gene in cultured human pituitary tumour cells with the hGH gene trans-

ected into rat cell lines and found differences in  $T_3$  regulation, while insulin and glucocorticoid regulation was similar: does this imply a tumour-specific abnormality in GH gene control? Comparison of normal pituitary tissue with adenoma tissue may answer this question, and quantitative in situ hybridization analysis should prove useful in further studies of small tissue samples (Pixley et al, 1987; Levy and Lightman, 1988).

Abnormalities at the level of the hGH gene itself have been proposed by U et al (1988) who found evidence for hypomethylation of the hGH gene and its 5'-flanking region in adenomas compared to normal pituitary tissue: gene hypomethylation is generally associated with enhanced expression in tissues where the genes are normally expressed (Razin and Riggs, 1980; Compere and Palmiter, 1981; Hjelle et al, 1982; Durrin et al, 1984; Laverriere et al, 1986).

U et al (1988) also found that the *c-fos* proto-oncogene was amplified in a single prolactinoma, which is of interest in that *c-fos* has been implicated in a mitogenic action of GRF and cyclic AMP in somatotrophs (Billestrup et al, 1987), and may modulate the DNA binding of the transcription factor AP-1 (Chiu et al, 1988). The role of such transcription factors in pituitary tumour development has yet to be addressed, but abnormalities in their DNA binding or in their phosphorylation by kinases may clearly have major effects on the level of gene transcription, and may be further sites of gene dysregulation involved in adenoma formation. The recent cloning and sequencing of cDNAs coding for the pituitary-specific transcription factor Pit-1/GHF-1 (Bodner et al, 1988; Ingraham et al, 1988) will undoubtedly stimulate investigation into the potential interactions between *trans*-acting factors in pituitary tumours.

Finally, pituitary tumours may be generated in transgenic mice. Massive lactotroph tumours have been induced in transgenic mice expressing a fusion gene consisting of 3000 bp of the PRL 5'-flanking region linked to the SV40 T antigen coding region (Rosenfeld et al, 1987), indicating that the malignant transformation induced by the SV40 gene was targeted exclusively to lactotrophs by the PRL 5'-flanking DNA. Mayo et al (1988) linked the GRF structural gene to a metallothionein promoter sequence, and found that the chronic high levels of GRF caused massive somatotroph hyperplasia. Interestingly, the pituitary was a major site of transgene expression in these mice, in contrast to other metallothionein-promoter transgenes, and GRF was co-produced with GH in many cells, suggesting that the transgenic pituitaries might have developed autocrine regulation by GRF. The development of a GH-secreting tumour in a patient who had long-standing ectopic GRF secretion (Asa et al, 1984) indicates a possible pathogenetic role for abnormal (possibly autocrine) expression of GRF.

## CONCLUSIONS

The prolactin and GH genes are related and their structure and regulation have been extensively studied in rat pituitary cell systems. Mechanisms for their tissue-specific expression and hormonal regulation are rapidly being



elucidated, and a complex picture of inter-related nuclear protein/proto-oncogene effects on the genes is developing. The recent identification and cloning of such *trans*-acting factors as Pit-1/GHF-1 and AP-1 are major advances in the field, and will undoubtedly clarify our understanding. The pathogenesis of pituitary tumours remains a challenge, and has recently stimulated the application of molecular biological techniques and knowledge to human pituitary tissue. Whether tumour development can be attributed to specific defects at the level of hormone gene regulation will be the next exciting question that must now be addressed.

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# Giant Invasive Prolactinoma: A Case Report and Review of Nine Further Cases

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## SUMMARY

Very large prolactinomas are rare and may be difficult to treat. We present the report of a patient with an exceptionally aggressive tumour which extended outside the skull and emerged within the internal jugular vein. Bromocriptine induced only transient suppression of serum prolactin. *In vitro* studies of tissue from this prolactinoma showed that although prolactin secretion was reduced by both bromocriptine and dopamine, neither agent affected cytoplasmic levels of prolactin mRNA, suggesting relative autonomy of prolactin synthesis.

We reviewed the progress of nine other patients with very large prolactinomas and serum prolactin levels  $> 100,000$  mU/l. In three cases, treatment with bromocriptine rapidly reduced tumour size and serum prolactin levels became normal, but in the other cases responses were slower and less marked. Substantial amounts of residual pituitary tumour were seen in all cases where serum prolactin remained above 1000 mU/l, but also in one case where serum prolactin returned to normal, showing that serum prolactin levels are not reliable indicators of tumour size. Two patients died: one developed a CSF leak, and one pneumococcal meningitis in the absence of recognized CSF leakage. This emphasizes the significant morbidity associated with very large prolactinomas. The ideal long-term treatment of these tumours remains uncertain, but bromocriptine therapy alone is often inadequate. Cases of apparent bromocriptine resistance may reflect abnormal regulation of prolactin gene expression.

## INTRODUCTION

Prolactin-secreting pituitary tumours in women commonly present as small intrasellar microadenomas, but in men, the tumours may be much larger, and present as macroadenomas of more than 1 cm diameter [1, 2]. 'Giant' prolactinomas, several centimetres in diameter show massive extrasellar extension, and appear to be rare: they may provide special problems of management. One such case from this department [3] showed dramatic shrinkage over six days of bromocriptine therapy, although this was complicated by CSF rhinorrhoea requiring lumbo-peritoneal shunting. A further report described two tumours  $> 4$  cm diameter treated with bromocriptine [4]: one tumour was only partially responsive to

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this therapy, as measured by both serum prolactin levels and tumour size, although the other tumour had responded satisfactorily about eight years after radiotherapy.

We present the case of a man with a large prolactinoma whose tumour was locally aggressive, extending through the inferior petrosal sinus to emerge within the internal jugular vein. Serum prolactin levels rose above 100,000 mU/l and were only partially responsive to treatment with bromocriptine. Tissue from this tumour was examined *in vitro* to assess regulation of prolactin secretion and mRNA levels by bromocriptine. We also review our experience with the nine other cases of giant prolactinomas with serum prolactin levels > 100,000 mU/l seen in this department within the past five years.

## CASE REPORT

### *Patient 1. MH, male, 45 years old*

This man presented in 1982 at the age of 40 years with a six-week history of frontal headache, diplopia and right-sided ptosis. He had been previously fit and healthy, and took no medication. He was single and had no children. On examination, he had partial right 3rd, 4th and 6th cranial nerve palsies, and there was partial loss of the nasal field of vision in the left eye. Skull radiography showed an expanded pituitary fossa, and CT scan revealed a tumour extending laterally and into the right sphenoid sinus, but with little suprasellar extension. Endocrine testing pre-operatively revealed a serum prolactin of 91,600 mU/l (normal < 500 mU/l), and total thyroxine of 73 nmol/l (normal 60–140 nmol/l); 9 a.m. plasma cortisol was 260 nmol/l, testosterone was < 0.6 nmol/l (normal 10–30 nmol/l) with luteinizing hormone and follicle stimulating hormone levels of 3 and 4 U/l, respectively. A frontal craniotomy was carried out and the lack of suprasellar extension was confirmed. All of the intrasellar tumour was removed, but some of the laterally extending tissue was not accessible. Histological examination of the pituitary tissue revealed excessive nuclear variation with a large number of mitoses.

Post-operatively, the patient's visual fields became normal, but the 3rd, 4th and 6th cranial nerve palsies remained. Serum prolactin levels fell only slightly, but he became panhypopituitary. He was treated with bromocriptine 7.5 mg daily and hydrocortisone, thyroxine and testosterone replacement therapy. He then received 4500 cGy external irradiation to the pituitary region (three fields) in 30 fractions over a six-week period. Serum prolactin levels fell to 11,000 mU/l after two months on bromocriptine 7.5 mg daily, but over the next nine months gradually rose to > 39,000 mU/l, despite increasing the drug dose to 15 mg daily (Figure 1A).

Bromocriptine was discontinued during 1984 in view of its lack of sustained effect on serum prolactin levels, but he remained well over the next two years.

In April 1986, he developed nausea and vomiting, and was found to be dysarthric, and to have right-sided 9th, 10th and 12th cranial nerve palsies, in addition to his pre-existing 3rd, 4th and 6th nerve palsies. CT scans (Figure 2) showed little change compared to 1982, but the tumour was assumed to have extended inferiorly, causing lower cranial nerve damage. Serum prolactin was 120,000 mU/l. A further trial of bromocriptine, up to 30 mg daily was commenced. Although this reduced prolactin levels to 20,000 mU/l after eight weeks (Figure 1B), the patient experienced severe nausea on the highest doses, and in June 1986 he underwent a transethmoidal hypophysectomy in an attempt to reduce the tumour bulk. Most of the large tumour mass, which extended laterally to the right, superiorly and inferiorly, was removed. Histological examination showed a cellular chromophobe neoplasm composed of solid sheets of pleomorphic cells with hyperchromatic nuclei and a

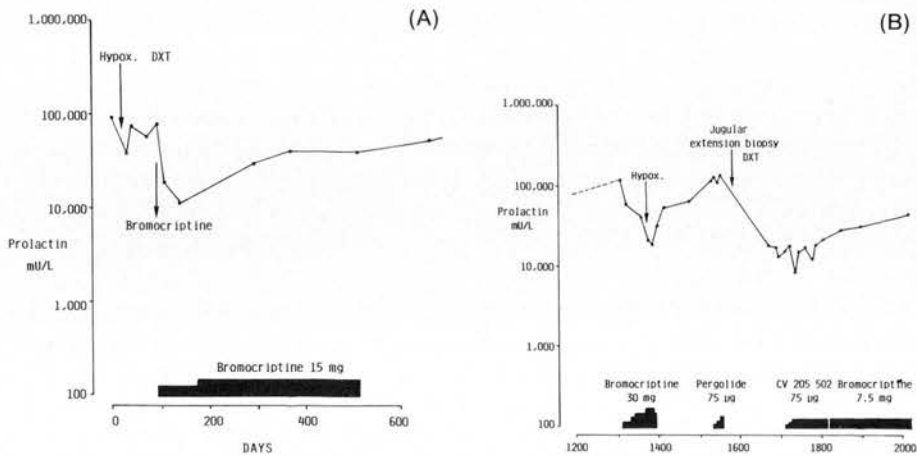


FIG. 1. A. Case 1, MH: initial response of serum prolactin to surgery, radiotherapy and bromocriptine, days 0–600. B. Later responses of serum prolactin to bromocriptine, pergolide and CV 205-502, over days 1200–2000. Note serum prolactin shown on a logarithmic scale.

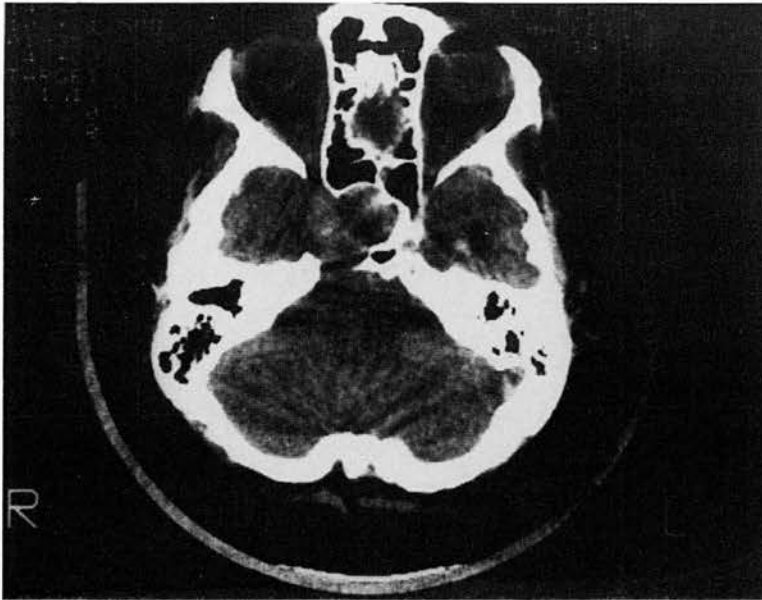


FIG. 2. Case 1, MH: CT scan at the level of the floor of the pituitary fossa in 1986, showing extensive bony destruction and right inferior and parasellar extension.

variable amount of cytoplasm. Numerous binucleate cells and mitotic figures were found. Tumour tissue obtained at this operation was used for *in vitro* studies of prolactin release and mRNA accumulation.

Post-operatively, the patient continued to suffer from severe nausea, and as a result the bromocriptine was stopped. Serum prolactin levels began to rise again (Figure 1B), but he remained otherwise well until December 1986 when he developed worsening headache, nausea and deterioration of his diplopia. Other neurological signs were unaltered.

In view of a report that some patients with bromocriptine-resistant prolactinomas may

respond to pergolide [5], this treatment was started, but doses up to 75 µg daily had no effect on prolactin levels over a three-week period (Figure 1B) and it was therefore stopped. One week later he developed a right parapharyngeal abscess, which was treated successfully with antibiotics. A residual hard lymph node was found at the angle of the right mandible, and excision biopsy revealed a lymph node adjacent to a mass of tumour tissue within the internal jugular vein. Histological examination revealed only reactive changes in the lymph node, but confirmed infiltration of the vein wall with pleomorphic cells which showed positive immunostaining for prolactin. A fragment of this tissue secreted prolactin when cultured *in vitro* (data not shown).

The patient was given further radiotherapy to the neck and skull base, receiving 3000 cGy in 10 fractions over 14 days, in an effort to halt extracranial extension of the pituitary tumour, and serum prolactin levels fell markedly over the next three months from 140,000 to 18,000 mU/l, in the absence of any further bromocriptine therapy. Four months later a CT scan suggested a substantial mass of tumour remaining in the inferior petrosal sinus, and he was therefore offered a trial of the new non-ergot dopamine agonist drug CV 205-502 (Sandoz Pharmaceuticals). Although acute administration of this agent caused transient suppression of serum prolactin within 24 hours (from 18,000 to 11,000 mU/l) this suppression was not sustained with chronic treatment over the subsequent three months, and serum prolactin levels remained between 12,600 and 22,100 mU/l (Figure 1B). The patient reverted to bromocriptine treatment (7.5 mg daily), but his prolactin levels continued to rise gradually to > 46,000 mU/l in 1988. He complained of facial pain at this time, and clinical examination suggested tumour involvement of the right fifth cranial nerve. A third hypophysectomy was performed in a further attempt to reduce the tumour bulk, and this resulted in complete relief of the facial pain.

## CELL CULTURE STUDIES

### *Methods*

Prolactinoma tissue obtained at the second hypophysectomy was grown in monolayer culture for *in vitro* studies of the regulation of prolactin release and prolactin mRNA accumulation. These experimental data have previously been presented as part of a separate report [6]. Pituitary tumour tissue was enzymatically dispersed using collagenase and hyaluronidase, as previously described for rat pituitary tissue [7]. Dispersed prolactinoma cells were plated onto plastic cell culture dishes at a density of approximately  $2 \times 10^5$  cells/2 ml/well in final culture medium (Dulbecco's modified Eagle's medium supplemented with 10% horse serum, 2.5% fetal calf serum and 2 mM glutamine). Cells were allowed to attach to the plastic dishes and were grown in monolayer culture for five days. They were then washed by repeated medium exchanges, and treated with bromocriptine or dopamine in serum-free medium for 24 hours. Medium was then removed for radioimmunoassay of released hormone, using MRC 83/562 as prolactin standard, and MRC 66/217 as standard for growth hormone. Meanwhile cytoplasmic extracts were prepared for dot hybridization analysis, which was carried out as described previously [7] using a  $^{32}\text{P}$ -labelled cDNA probe specific for human prolactin [8], generously provided by Dr J. A. Martial, University of Liège, Belgium.

## RESULTS

Over the first four days in culture the cells produced large quantities of prolactin, and very small amounts of growth hormone (data not shown); however growth hormone secretion

was not detectable in subsequent 24-hour incubations, implying that any contamination with normal pituitary tissue was negligible. Treatment for 24 hours with either 10 nM or 100 nM bromocriptine reduced prolactin release from the cultured cells to approximately 50% of control values; 10  $\mu$ M dopamine had a similar effect. On the other hand, prolactin mRNA levels were only minimally reduced by bromocriptine, and a single high dose of dopamine had no effect (Figure 3). These data contrast with those found with normal rat pituitary tissue, where clear inhibition of prolactin mRNA accumulation by similar doses of bromocriptine can be demonstrated [9]. A single high dose (100 nmol/l) of thyrotrophin-releasing hormone stimulated prolactin release, but had only marginal and non-significant effects on prolactin mRNA levels, again in contrast to results obtained with normal rat pituitary tissue (data not shown).

## REVIEW OF NINE FURTHER CASES OF GIANT PROLACTINOMA

The unusual problems encountered with this patient prompted us to review the experience in this department with other cases of 'giant' prolactinomas. Nine other patients were identified from the endocrine clinic in whom serum prolactin had exceeded 100,000 mU/l, as an arbitrary criterion for massive lactotroph hyperplasia. Their cases are briefly summarized below and in Table 1, and initial responses to bromocriptine are shown in Figure 4 in approximate order of their responsiveness to bromocriptine. Four of the patients received bromocriptine alone, but five were treated surgically before receiving the drug. Only one patient (case 4) also had radiotherapy.

## CASE REPORTS

### *Patient 2. EO, female, 60 years old*

This patient has been reported in detail previously [3]. She presented in 1983 at the age of 55 years with visual deterioration, headache, loss of memory and long-standing amenorrhoea. Her initial serum prolactin level was 696,000 mU/l. She had bitemporal hemianopia and cranial CT scanning revealed a massive pituitary tumour extending upwards into the middle and anterior cranial fossae, inferiorly into sphenoid and ethmoid sinuses, and posteriorly along the skull base. Bromocriptine at doses reaching 30 mg/day, induced a dramatic fall in

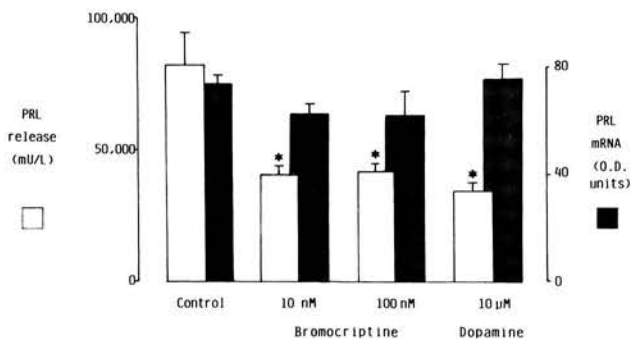


FIG. 3. *In vitro* responses of cultured prolactinoma cells from patient MH: prolactin release (open bars) and cytoplasmic prolactin mRNA concentrations (stippled bars), in response to 10 and 100 nM bromocriptine, and 10  $\mu$ M dopamine. Data shown are means  $\pm$  SEM,  $n = 4$  per group and mRNA data are shown in arbitrary units of optical density; \* $2P < 0.002$  compared to respective controls.

TABLE 1. *Summary of initial and final serum prolactin levels, CT scan outcome, and complications in giant prolactinoma cases*

Patient	Serum prolactin (mU/l)		Final CT appearance	Bromocriptine maintenance dose (mg) (maximum)		Complications
	Initial	Final (time)				
1. MH	91,600	46,000 (6 years)	Extensive skull base erosion	7.5	(30)	Local extension
2. EO	696,000	450 (5 years)	Empty sella	5	(30)	CSF leakage
3. JW	270,000	350 (4 years)	Persistent sphenoid sinus extension	10	(10)	—
4. AH	290,000	560 (2 years)	Partial regression but persistent suprasellar tumour	5	(10)	Sudden death (2 years); uncertain cause
5. FK	119,750	730 (4 years)	Small intrasellar tumour residue	5	(15)	—
6. GJ	450,000	4900 (1 year)	Extensive supra- and para-sellar persistent tumour	20	(25)	Pneumococcal meningitis
7. JB	141,800	1200 (7 years)	Persistent tumour extension into sphenoid sinus	17.5	(25)	—
8. GA	107,800	3900 (2 years)	Partial regression but persistent suprasellar tumour	5	(5)	—
9. RP	170,000	21,000 (5 years)	Persistent inferior and posterior extension	20	(20)	—
10. AC	160,000	55,000 (15 days)	No change in tumour volume at 15 days	10	(10)	Death (15 days); presumed pituitary infarction

serum prolactin levels, to 3010 mU/l after three weeks, by which time her visual fields were normal, and CT scanning showed rapid shrinkage of the tumour. Prolactin levels were normal (<450 mU/l) by four months after bromocriptine treatment. The only complication of treatment was CSF rhinorrhoea which developed as the tumour shrank. Nearly five years later she remains well with normal serum prolactin levels on bromocriptine 5 mg daily. A CT scan in 1987 showed a largely empty pituitary fossa.

*Patient 3. JW, male, 67 years old*

This man presented in 1984 with visual failure and hypogonadism. His initial serum prolactin was 270,000 mU/l, and a CT scan showed a large pituitary tumour extending through the pentagonal cistern to indent the inferior aspect of the third ventricle; the tumour extended 5 cm superiorly, and 4 cm transversely. The patient was treated with bromocriptine in doses of up to 10 mg daily, and serum prolactin levels fell below 1000 mU/l within two months; after 4 years serum prolactin remains at 330–470 mU/l. However repeat CT scanning showed that despite significant reduction in the suprasellar mass, persistent tumour

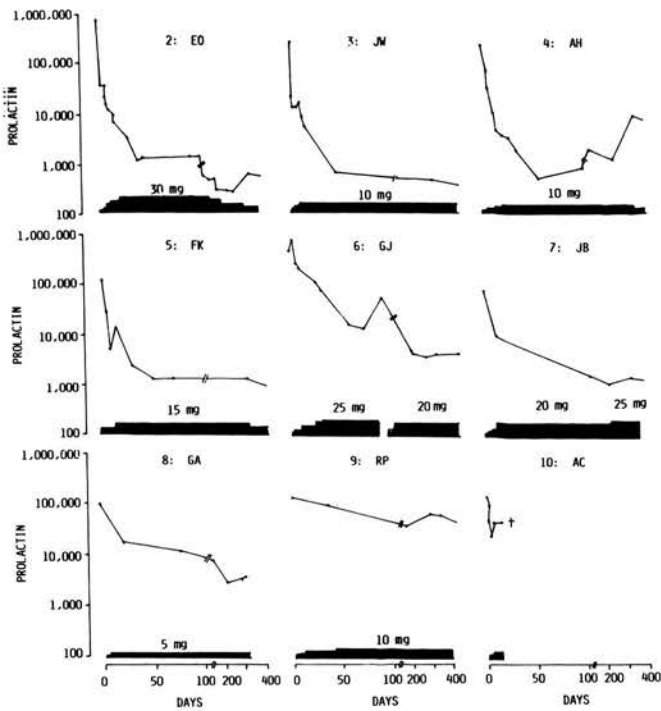


FIG. 4. Summary of nine cases of giant prolactinoma: initial responses of serum prolactin to bromocriptine over days 0–400. Longer term outcome for each patient is summarized in Table 1. Doses of bromocriptine are shown by hatched bars, and serum prolactin is shown on a logarithmic scale.

still extends into both sphenoid sinuses, partly destroying the dorsum sellae and posterior clinoid processes, implying a large tumour mass despite normal prolactin levels in the peripheral blood.

#### *Patient 4. AH, male, 34 years old*

This man presented in 1984 with a history of right-sided headaches, and symptoms of hypogonadism. He had first presented to a neurosurgical unit in 1970 with headaches and visual field loss. A large pituitary chromophobe adenoma was removed at transfrontal hypophysectomy, and he subsequently received pituitary radiotherapy. He remained well on steroid and thyroxine replacement therapy until 1984 when he developed further headaches. Serum prolactin was 290,000 mU/l, and testosterone was undetectable. CT scanning showed a large enhancing mass with parasellar extension, suprasellar extension with a total vertical extent of 4.5 cm, and erosion of the petrous temporal apex. Bromocriptine, increased to 10 mg daily, induced a rapid reduction in serum prolactin (Figure 4). When the dose of bromocriptine was reduced to 2.5 mg daily, prolactin levels rose to 42,200 mU/l, but on raising the dose to 5 mg daily, serum prolactin fell to 560 mU/l, and the patient was well and symptom-free. The most recent CT scan, in 1986, showed some reduction in tumour size, but persistence of moderate suprasellar extension. The patient died suddenly in early 1987, and no obvious cause was found at post-mortem, apart from the presence of the pituitary tumour.



*Patient 5. FK, male, 54 years old*

This man presented in 1984 at the age of 50 years, with a three-week history of headache, decreasing conscious level and left-sided weakness. A CT scan showed a large cystic lesion in the right frontal and central areas, arising from the pituitary fossa region, with enhancing tissue at its base. A cystic tumour was aspirated at craniotomy with clinical improvement. Serum prolactin was 154,000 mU/l initially and 119,000 mU/l post-operatively; this fell progressively with bromocriptine therapy, reaching 1300 mU/l at eight weeks and eventually becoming normal nearly two years after treatment started (Figure 4). He remains well after four years of 10 mg bromocriptine treatment. CT scans two years after treatment demonstrated that the tumour had largely regressed to a small area within the pituitary fossa, with only minimal parasellar extension still present.

*Patient 6. GJ, male, 22 years old*

This patient presented in 1985 at the age of 19 years with a 15-month history of progressive visual loss. He was obese and clinically hypopituitary, and CT scanning showed a large lobulated suprasellar mass extending approximately 4 cm into the middle cranial fossa, and also extending inferiorly into the sphenoidal and ethmoidal sinuses and nasopharynx, destroying much of the base of the skull. A large tumour mass was partly excised at craniotomy. Post-operatively, serum prolactin levels were between 450,000 and 1,652,000 mU/l, and the patient was referred for medical treatment of his prolactinoma. Bromocriptine (2.5 mg) reduced serum prolactin to 190,500 mU/l over an initial six-hour period; continuing bromocriptine treatment (up to 25 mg daily) over the subsequent eight weeks progressively reduced serum prolactin levels to 13,500 mU/l (Figure 4).

At this stage, the patient developed pneumococcal meningitis which was successfully treated with penicillin. Although he had never developed CSF rhinorrhoea, a defect in the skull base was the presumed portal of bacterial entry. With subsequent bromocriptine therapy (20 mg daily), prolactin levels fell to 3900 mU/l, but have not normalized during a total treatment period of 18 months. CT scanning 15 months after presentation showed some reduction in size of the tumour in the middle cranial fossa, but persistence of large inferior, posterior and parasellar extensions of the tumour.

*Patient 7. JB, male, 35 years old*

This man presented in 1981 with obesity, and was found to be hypopituitary. Serum prolactin was initially 141,600 mU/l, and CT scanning revealed a large pituitary tumour with downward extension to the left and 2 cm suprasellar extension. Visual fields were normal. With bromocriptine (up to 25 mg daily), prolactin levels fell from 84,800 mU/l to approximately 1000 mU/l (Figure 4). Seven years after diagnosis serum prolactin levels have become stable at 1200–1900 mU/l; however repeat CT scans show that, despite regression of the suprasellar component, large amounts of pituitary tumour persist with extension into the upper part of the left sphenoid sinus, and erosion of the dorsum sellae.

*Patient 8. GA, male, 40 years old*

This man presented in 1986 at the age of 39 years with grand mal convulsions. A CT scan showed a pituitary tumour with an irregular enhancing suprasellar mass. This was subtotally removed at a craniotomy; histological examination showed a cellular tumour,

staining strongly positive for prolactin. The post-operative serum prolactin level was 113,100 mU/l and after starting bromocriptine (5 mg daily) there was a progressive fall to 3,900 mU/l over the next 12 months (Figure 4). CT scanning approximately 15 months after treatment revealed a small amount of suprasellar enhancing tissue, implying partial regression of the tumour. There have been no complications over the 18 months to date.

*Patient 9. RP, male, 41 years old*

This patient presented in 1983 with a two-year history of headache. CT scanning showed a pituitary tumour with modest suprasellar extension to 0.5 cm above the fossa, but marked left parasellar involvement. Serum prolactin level was 170,000 mU/l; this was reduced to 46,000 mU/l after three months' bromocriptine therapy, at which time a transethmoidal hypophysectomy revealed a largely empty sella. Subsequent CT scans confirmed low density within the pituitary fossa, but demonstrated persistent posterior and parasellar extension of the tumour.

In December 1984 the patient recommenced long-term bromocriptine treatment (10 mg daily), and prolactin levels fell very slowly from 107,000 to 70,800 mU/l at the end of the first year (Figure 4). There was a further fall, to 23,500 mU/l after nearly three years of maintenance treatment with 20 mg bromocriptine daily, but recent CT scans have shown only minimal reduction in size of the tumour.

*Patient 10. AC, male, 65 years old*

This patient presented with collapse, possibly a convulsion, after which he was found to have inappropriate secretion of anti-diuretic hormone. He had complained of impotence for over 40 years, and on examination he was clinically hypopituitary. A CT scan showed a large pituitary tumour, and a serum prolactin level was 160,000 mU/l. During this admission the patient suddenly collapsed again, becoming unrousable and mute; he had neck stiffness and deviated gaze, and then had one grand mal convulsion. A clinical diagnosis of acute pituitary infarction was made. Treatment with dexamethasone and bromocriptine resulted in an initial improvement, with regained consciousness and a fall in serum prolactin levels; however a repeat CT scan 10 days after starting bromocriptine showed no change in the size of the tumour. His condition deteriorated during the second week and he died 15 days after bromocriptine therapy commenced. A post-mortem examination was not performed.

## DISCUSSION

We have presented the cases of ten patients with extremely high serum prolactin levels who could be classed as having giant prolactinomas. The index case (MH) had an unusually aggressive and locally invasive tumour which appeared to be only partly responsive to treatment with bromocriptine. The tumour was particularly unusual in its spread along the skull base, with local extension through the inferior petrosal sinus; to our knowledge, this is the first case where prolactinoma extension outside of the skull has been reported. The aggressive nature of the tumour was suggested by the numerous mitotic figures in the original tissue. Intracranial metastases from prolactinomas have been described [10–13] but appear to be exceptional, and in the absence of distant metastasis this patient's tumour cannot truly be classed as a carcinoma.

This tumour appeared to be partly responsive to dopamine agonist therapy, since acute challenges with bromocriptine, pergolide and CV 205-502 resulted in transient suppression



of serum prolactin levels; however such suppression was not sustained, and prolactin levels rose despite continuing bromocriptine treatment on two separate occasions. Failure of bromocriptine to suppress prolactin secretion completely in a proportion of cases is well recognized [14–16], although the reasons why tumours differ in this way are not clear. The basis for dopamine resistance is not understood, but rat tumour models suggest a loss of dopamine receptors or loss of receptor-associated G-proteins, which may be involved in both dopamine and thyrotropin releasing hormone action [17]. The *in vitro* study showed 50% inhibition of prolactin release by high doses of bromocriptine and dopamine, implying intact receptor and post-receptor function, but there was no significant effect on cytoplasmic accumulation of prolactin mRNA. These data therefore suggest relative autonomy of pre-translational stages of prolactin synthesis in this tumour, and it may be that this underlies the lack of sustained prolactin suppression in this patient. Similar *in vitro* studies of tumour somatotrophs removed at hypophysectomy from patients with acromegaly have also suggested variable and abnormal mRNA regulation [18], and further studies of gene expression in these tumours are likely to clarify potential pathological mechanisms.

Review of the other nine cases of 'giant prolactinomas' suggests that this patient's tumour was unusually aggressive, even considering its extent. All but one of the other patients responded to bromocriptine with at least some suppression of serum prolactin levels, but the speed and degree of response was variable. Three patients (EO, JW and AH) showed rapid and nearly complete responses to bromocriptine, reduction of serum prolactin to normal within four months; only one of the other patients' prolactin levels became normal after two years of treatment (FK), while in three cases (GJ, JB and GA), prolactin levels remained static between 1200 and 3900 mU/l at one to seven years after initial treatment. Patient AC died before any assessment could be made of his likely long-term response to treatment. The only patient who showed marked resistance to bromocriptine was RP, although even this man has shown sustained partial suppression of serum prolactin on high doses, in contrast to the index case. There was no apparent correlation between the dose of bromocriptine used and the ultimate response in these patients, although a rapid initial fall in prolactin levels appeared to favour better long-term control.

Serum prolactin is often regarded as an approximate marker of prolactinoma volume, and it appears that incomplete suppression of serum prolactin by bromocriptine may imply the persistence of substantial amounts of residual tumour. However, in one patient (JW) a large tumour mass persisted despite normalization of serum prolactin. A previous report of two large invasive prolactinomas describes one case where a 95% reduction in serum prolactin levels was associated with only 20% reduction in tumour size [4], and, together with our data, this suggests that serum prolactin level may not be a reliable marker of tumour size [19].

The ideal management for patients with large prolactinomas remains uncertain [1, 4]. Estimation of serum prolactin is essential before proceeding to surgical treatment, and a trial of dopamine agonist therapy should be considered, as these patients will not be cured by surgery alone. Bromocriptine will induce at least 50% reduction in the initial volume of prolactin-secreting macroadenomas in about two-thirds of cases [2, 20], but the very large tumours may differ. The present series indicates that patients with very large prolactinomas may suffer serious morbidity (MH and GJ), and indeed two patients in this series (AH and AC) died suddenly; one death (AH) remains unexplained, and the other patient (AC) is thought to have suffered pituitary infarction before bromocriptine treatment was started. Bromocriptine was clearly able to correct serum prolactin levels in some patients, although the speed and extent of response was highly variable, and normal prolactin levels were not achieved for nearly two years in one case.

The policy in this unit has been to use low doses of bromocriptine as long as there is a

continuing reduction in the level of serum prolactin, and then increase doses to the maximum that the patient can tolerate. However the ideal drug regimen remains to be established in such cases; the persistence of large tumour masses despite normal levels of serum prolactin may possibly be related to slow response, and this could argue for more aggressive initial treatment. Special problems with bromocriptine treatment in these patients appeared to relate mainly to the risk of skull base defects appearing once tumour tissue had regressed, either causing CSF rhinorrhoea, as is well recognized [21], or allowing CSF infection. Long-term treatment with bromocriptine may be well tolerated and maintain clinical remission even in cases of very large prolactinomas, but a rebound rise in serum prolactin and re-expansion of tumour usually occurs on stopping the drug [22]. The documented persistence of tumour tissue may make pituitary megavoltage radiotherapy advisable for long-term control of the disease [2]. In conclusion, large prolactinomas may be very aggressive and cause serious morbidity or even death, and they may not always respond adequately to bromocriptine. Their treatment requires close supervision, with an awareness of the potential serious complications.

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## Suppression of prolactin release and mRNA accumulation by two novel dopamine agonist agents

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**Abstract.** Two novel dopamine agonist drugs, CV 205-502 and CQP 201-403, have been investigated to compare their effects on prolactin secretion and prolactin mRNA accumulation in cultured rat pituitary cells. Both drugs gave dose-dependent suppression of prolactin release over a 24 h incubation period: when each drug was used at 100 nmol/l CV 205-502 and CQP 201-403 induced suppression to  $8.9 \pm 1.7$  and  $10.2 \pm 1.8\%$  of control release, respectively, compared to  $26.7 \pm 4.8\%$  of control with 100 nmol/l bromocriptine. There was no consistent effect on growth hormone release. Cytoplasmic accumulation of prolactin mRNA was also inhibited by both drugs at this concentration, to  $50.2 \pm 5.5\%$  of control values by CV 205-502 and to  $67.4 \pm 8\%$  of control by CQP 201-403, and to a similar extent by 100 nmol/l bromocriptine ( $50.6 \pm 9.1\%$  of control). None of the drugs had any significant effect on GH mRNA levels. These data suggest that the agents exert their effect at a pre-translational stage of prolactin synthesis, as well as at the level of hormone release.

Prolactin synthesis and release are under the control of a number of different hormones, but the dominant inhibitory influence in vivo is thought to be exerted by dopamine (Franks & Jacobs 1983). Dopamine acts in vitro to inhibit prolactin gene transcription and hence reduce accumulation of prolactin messenger RNA (mRNA) in cultured pituitary cells (Maurer 1980), and the dopamine agonist drug bromocriptine has a similar effect (Maurer 1981). The prolactin-inhibitory effect of bromocriptine has been of dramatic benefit clinically

in the treatment of prolactin-secreting pituitary tumours; in addition to reducing serum prolactin levels, both bromocriptine and the newer ergot derivative pergolide have been shown to reduce the size of large prolactinomas (Kendall-Taylor et al. 1982; Franks & Jacobs 1983).

The use of these ergot-derived agents has become well-established, but the frequency of adverse effects has led to the recent development of two new agents, CV 205-502 and CQP 201-403 (Sandoz Pharmaceuticals). CQP 201-403 is a propylergoline and CV 205-502 a benzo(g)quinoline (Nordman & Petcher 1985). Both drugs have been shown to be active at lower doses than bromocriptine in vivo in both normal subjects and in hyperprolactinemic women (Grevel et al. 1986; Rasmussen et al. 1987; Hanssen et al. 1988), and so far they appear to have few adverse effects.

Both agents exert inhibitory effects on prolactin release by normal rat pituitary tissue and also human prolactinoma tissue (Venetikonou et al. 1987). These studies showed that in comparison to the transient inhibitory effect of dopamine, both CV 205-502 and CQP 201-403 caused profound and prolonged inhibition of prolactin secretion, while there was little or no effect on growth hormone release.

Although it is therefore clear that these agents reduce prolactin release, there is so far no information as to possible effects on hormone synthesis,

which is of some importance if the drugs are to be of value in the long-term treatment of hyperprolactinemia or in attempts to reduce the size of large prolactinomas. We have therefore assessed the effects of these two drugs on the pre-translational stages of prolactin synthesis by measuring cytoplasmic accumulation of prolactin and growth hormone (GH) mRNA in cultured rat pituitary cells.

## Materials and Methods

### Cell culture

Anterior pituitary glands were removed from male Wistar rats and were enzymatically dispersed as described previously (Davis & Sheppard 1986a). Cells were plated onto 24-well culture plates at a density of approximately  $0.5 \times 10^6$  cells/well in final culture medium which consisted of Dulbecco's modified Eagle's medium with 0.3% bovine serum albumin, supplemented with 10% horse serum, 2.5% fetal calf serum and 2 mmol/l glutamine. Cells were allowed to attach to the dishes and were grown in monolayer culture for 3–4 days before experiments. They were then transferred to serum-free medium, washed by repeated medium exchange, and treated for 24-h periods with either CV 205–502, CQP 201–403 or bromocriptine (Sigma Chemical Company, Poole, Dorset, UK). All three drugs were initially dissolved in ethanol, before dilution in medium to the final concentrations indicated. At the end of treatment periods, medium was removed for radioimmunoassay of released hormone, and cells were scraped from culture wells for preparation of cytoplasmic samples as described previously (Davis & Sheppard 1986b).

### Messenger RNA measurements

Cytoplasmic accumulation of prolactin or GH mRNA was measured by a cytoplasmic dot hybridization technique (White & Bancroft 1982), as described previously (Franklyn et al. 1986). Briefly, samples of cytoplasmic extract from the cultured pituitary cells were diluted and applied in duplicate to a hybridization transfer membrane (Gene Screen, New England Nuclear, Du Pont UK, Southampton, UK), and hybridized with  $^{32}$ P-labelled plasmid probes containing complementary DNA inserts specific for rat prolactin (pPRL-1; Gubbins et al. 1979) or rat GH (pBR322 rGH-1; Harpold et al. 1978) (kindly provided by Dr R A Maurer, University of Iowa, USA, and Dr F C Bancroft, Mt Sinai School of Medicine, New York, USA respectively). Probes were labelled by nick translation using commercial reagents (Amersham International, Amersham, Bucks, UK) to a specific activity of  $1-4 \times 10^8$  dpm/ $\mu$ g DNA. After hybridization, mRNA concentrations were measured by autoradiography and

scanning densitometry; mRNA concentrations are expressed in arbitrary units of optical density relative to control data which are given a value of 1.0 units.

### Radioimmunoassays

Prolactin and growth hormone release were measured by radioimmunoassay using reagents supplied by the National Hormone and Pituitary Program, NIADDK, Bethesda, MD. Prolactin results are expressed in terms of the standard rPRL-RP3, and GH in terms of standard rGH-RP2.

### Statistics

Statistical significance was calculated using one-way analysis of variance, and results were considered significantly different from control when *P* had a value less than 0.05.

## Results

Both CV 205–502 and CQP 201–403 caused similar dose-dependent inhibition of prolactin release in initial experiments to 8.9 and 13.6% of control release, respectively, at 100 nmol/l concentrations ( $P < 0.01$ ; Fig. 1). GH release in these experiments was somewhat variable but no consistent dose-dependent effect was seen ( $P > 0.05$ ; data not shown).

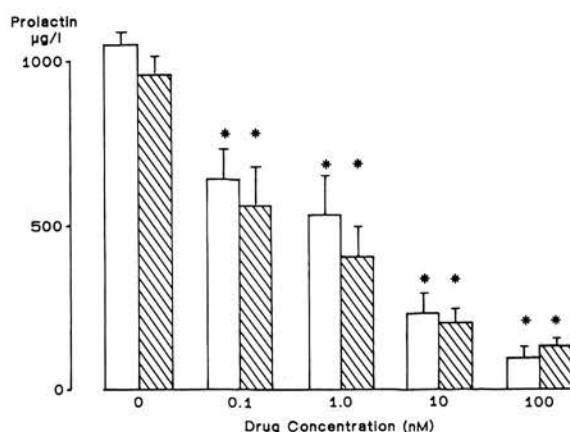


Fig. 1.

Effect of increasing doses of CV 205–502 (□) and CQP 201–403 (▨) on prolactin release from cultured rat pituitary cells. Data are means  $\pm$  SEM, *N* = 7 replicate culture wells, pooled from two separate experiments; \*:  $P < 0.01$  compared to control.



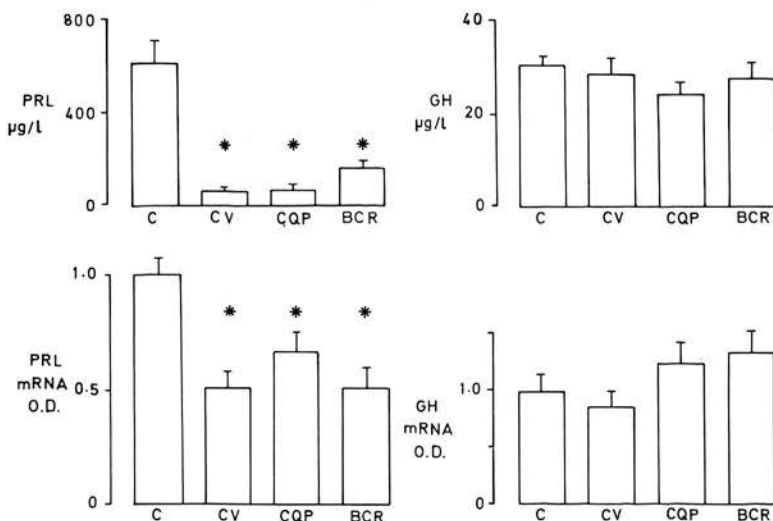


Fig. 2.

Effects of 100 nmol/l of CV 205-502, CQP 201-403 and bromocriptine on prolactin release, prolactin mRNA concentrations, GH release and GH mRNA concentrations. Messenger RNA data are expressed in arbitrary units of optical density (O.D.), relative to control values of 1.0. Data shown are combined results of 3 (prolactin data) or 2 (GH data) separate experiments,  $N = 8-18$  replicate culture wells for each group; \*:  $P < 0.005$  compared to control.

Both agents also inhibited prolactin mRNA accumulation. Dose-dependent inhibitory effects of each drug were variable and not readily reproducible between experiments, but statistically significant inhibition of prolactin mRNA levels (to 69% and 52% of control in single experiments) could be seen with 1 nmol/l doses of CV 205-502 and CQP 201-403 ( $P < 0.05$ ; data not shown). However, the maximal inhibition was in all experiments less than that seen for prolactin release, and the greatest degree of suppression seen in individual experiments was to 42% of control with 100 nmol/l CQP 201-403.

Because of individual variability between dose-response experiments, a comparison was made of maximally effective prolactin-inhibitory doses of CV 205-502, CQP 201-403 and bromocriptine using data pooled from three separate experiments; this confirmed the striking inhibition achieved by all three agents on prolactin release ( $P < 0.005$ ), slightly greater with CV and CQP than with bromocriptine; on the other hand, prolactin mRNA accumulation was definitely reduced, but only to  $50.2 \pm 5.5$ ,  $67.4 \pm 8.0$  and  $50.6 \pm 9.1\%$  of control values by CV 205-502, CQP 201-403 and bromocriptine, respectively ( $P < 0.005$ ; Fig. 2). In

contrast, no inhibitory effects were seen on either GH release or GH mRNA accumulation ( $P > 0.05$ ; Fig. 2).

## Discussion

These data show that both of these dopamine agonist drugs caused dose-dependent inhibition of secretion of prolactin but not growth hormone, and both agents also induced significant suppression of prolactin mRNA concentrations without effect on GH mRNA levels.

In contrast to the profound inhibitory effects of both agents on prolactin release, the inhibition of prolactin mRNA accumulation was less marked, but comparable to that achieved by a similar high (100 nmol/l) dose of bromocriptine. The reason for this lesser effect on mRNA levels may be the relatively long half-life of prolactin mRNA (Laverriere et al. 1983; Rosenfeld et al. 1987), which means that rapid changes in the rate of gene transcription have slow effects on the cytoplasmic accumulation of mRNA species. In previous studies of the effect of bromocriptine, 50% inhibition of prolactin mRNA accumulation was seen between 24 and 48

h, (similar to the present findings with CV 205–502 and CQP 201–403), though maximal suppression of mRNA levels took up to 96 h (Maurer 1980). The newer dopamine agonists are more potent than bromocriptine *in vivo* (Rasmussen et al. 1987; Hanssen et al. 1988); the prolactin mRNA data shown here do not provide clear evidence for greater potency at the level of gene regulation, and further studies of longer term effects of lower doses of these drugs may be necessary to detect differences between the potency.

Neither CV 205–502 nor CQP 201–403 had any significant effect on either GH release or GH mRNA levels in this study. One previous study of these drugs *in vitro* showed a small inhibition of GH release with CQP 201–403 and no effect with CV 205–502 (Venetikou et al. 1987), although both drugs at least partly blocked the stimulation of GH release by GHRF. The present data confirm previous findings from this laboratory that GH mRNA levels were unaffected by bromocriptine (Wood et al. 1987), as was found with both the new dopamine agonist drugs.

In conclusion, the present study demonstrates suppressive effects on lactotrope cells of two new dopamine agonist agents, both at the level of acute hormone release but also a pre-translational site of action, to reduce prolactin mRNA accumulation and hence prolactin synthesis. These results are comparable to previous data for bromocriptine, and encourage the hope that the drugs may prove to be of value not only in the treatment of hyperprolactinemia, but also possibly in inducing shrinkage of large prolactin-producing pituitary adenomas.

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## Commentary

# Tissue-specific regulation of prolactin gene expression

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Production of prolactin is very tissue-specific – the gene is present in all cells, but in the rat it is expressed only in the anterior pituitary gland, while in man it is also expressed at low levels in the decidualized endometrium. Much of the work on rat prolactin gene expression has been greatly facilitated by the availability of the rat pituitary tumour-derived GH cell line (including the GH<sub>1</sub>, GH<sub>3</sub> and GH<sub>4</sub> cell subclones) which produces both prolactin and growth hormone. In contrast, much less is so far known about the regulation of the human prolactin gene, due in part to the lack of readily available human pituitary tissue for in-vitro studies.

An increasing amount is known about hormonal and intracellular regulation of prolactin mRNA production, which has been reviewed elsewhere (Davis, Belayew & Sheppard, 1989). However, some of the most impressive and important recent advances have been concerned with the molecular mechanisms of tissue-specific expression of prolactin, and these will form the subject of this brief survey. The majority of the work relates to the rat prolactin gene, but some information is now emerging for the human gene, which will be discussed separately.

### Rat prolactin gene: upstream regulatory DNA sequences

A variety of studies have recently provided a general model for eukaryotic gene regulation in which diffusible nuclear protein transcription factors (*trans*-acting factors) interact with defined sequences in 5'-flanking DNA (*cis*-acting elements) to effect both tissue-specific and hormonal regulation of gene transcription (Mitchell & Tjian, 1989). An analysis of these DNA-protein interactions is essential to a detailed understanding of the mechanisms of gene regulation. In the case of prolactin, the cloning of the gene itself has revealed that upstream of the structural gene there is a long stretch of regulatory DNA which is essential for tissue-specific and hormonally regulated expression of the protein.

The function of 5'-flanking DNA from the rat prolactin gene has been assessed by transfection studies in which up to 3 kb of upstream DNA sequences were linked to a reporter gene whose activity was then measured in the transfected cells as a function of the promoter sequence activity. There are two distinct upstream enhancer regions (Fig. 1), the proximal region located between –422 and –36 base pairs (bp) upstream of the transcription initiation site and the distal one between –1831 and –1530 bp upstream (Nelson, Crenshaw, Franco *et al.* 1986). Although these studies showed that the distal enhancer region alone was responsible for the majority of the transcriptional activity of the intact 3 kb upstream region, subsequent reports have shown that the proximal enhancer region is able to confer tissue-specific expression in its own right (Gutierrez-Hartmann, Siddiqui & Loukin, 1987; Lufkin & Bancroft, 1987). For example, using the technique of in-vitro transcription, the prolactin gene proximal enhancer region (–425 bp) had transcriptional activity *in vitro* only when incubated with nuclear or whole cell extracts from pituitary cells, and not with extracts from non-pituitary tissue (Gutierrez-Hartmann *et al.* 1987). This suggested that 5'-flanking DNA sequences from the proximal enhancer region contained sufficient information to direct tissue-specific expression, and that they interacted with a nuclear transcription factor that was present only in pituitary tissue. In summary, therefore, both the proximal and distal enhancer regions are important for transcriptional activation of the gene, although both regions have enhancer activity in their own right.

### Protein-DNA interactions: Pit-1/GHF-1

Detailed assessment of protein-DNA interactions with the prolactin gene regulatory DNA was carried out using the DNase I footprinting technique in which DNA regions protected from DNase I digestion by protein binding can be visualized by gel electrophoresis of digestion products. Gutierrez-Hartmann *et al.* (1987) found that incubation with pituitary nuclear proteins gave rise to four protected regions within the

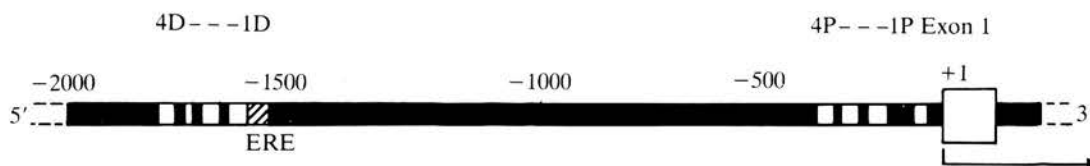


FIGURE 1. Diagrammatic representation of protein binding sites in the 5'-flanking DNA of the rat prolactin gene, with distances in base pairs upstream from the transcription initiation site, shown by the arrow. Proposed sites for the binding of the single protein factor Pit-1 are indicated by open regions; the four sites in the proximal enhancer region are indicated 1P–4P, and the four sites in the distal enhancer region 1D–4D. The oestrogen response element (ERE) is shown by the cross-hatched area in the distal enhancer region.

proximal 210 bp of the prolactin 5'-flanking DNA. One of these four sites was also seen with non-pituitary extracts, but the other three sites were pituitary cell-type specific. Similar footprinting studies were performed by Nelson, Albert, Elsholtz *et al.* (1988), who found that the distal enhancer region also contained four further sites which bound a pituitary-specific factor. These studies demonstrated mutual competition for protein binding between the different sites, implying that a single protein factor, named Pit-1, was involved. The rat growth hormone gene likewise contains two *cis*-acting elements which bind a pituitary-specific transcription factor, named GHF-1 (Bodner & Karin, 1987; Lefevre, Imagawa, Dana *et al.* 1987): interestingly, the studies of Nelson *et al.* (1988) showed that these sequences, like the prolactin sequences, also competed for factor binding to the prolactin promoter. Hence it was proposed that both the prolactin and growth hormone genes interacted with a common or functionally related pituitary-specific transcription factor(s). A number of different groups have now identified this factor for the rat prolactin gene using similar techniques (Cao, Barron & Sharp, 1988; Kim, Day & Maurer, 1988; Schuster, Treacy & Martin, 1988; Lufkin, Jackson, Pan & Bancroft, 1989).

DNA affinity purification using Pit-1 binding sites has shown that the protein is expressed as a 31 kD–33 kD doublet. The cDNAs for Pit-1 and GHF-1 were cloned independently by two groups in 1988 (Bodner, Castrillo, Theill *et al.* 1988; Ingraham, Chen, Mangalam *et al.* 1988), and it appears that the factors are indeed the same 33 kD protein. This protein contains an amino-terminal 60 amino acid region which is related to the 'homeodomain' which characterizes a class of genes important in embryonic development in *Drosophila*. At the carboxy-terminal a further 76 amino acid region is homologous to two other mammalian transcription factors, Oct-1, Oct-2, and the invertebrate protein factor *unc-86* (see Mitchell & Tjian, 1989 for review).

There is disagreement as to whether Pit-1/GHF-1 recognizes both the prolactin and growth hormone promoters, or only the growth hormone promoter

(Castrillo, Bodner & Karin, 1989; Mangalam, Albert, Ingraham *et al.* 1989). The mRNA for Pit-1/GHF-1 is expressed in lactotrophs and somatotrophs, but also in thyrotrophic cells (Crenshaw, Kalla, Simmons *et al.* 1989). The affinity purified Pit-1 protein is capable of directing transcription from both rat prolactin and growth hormone promoters *in vitro*, and permanent transfection studies have recently shown that expression of Pit-1 in non-pituitary HeLa cells, even at low levels, will direct transcription from both the prolactin and growth hormone promoters *in vivo* (Mangalam *et al.* 1989). Hence it appears that a single protein, found in three cell types in the anterior pituitary, can selectively activate transcription of either of two genes in their respective pituitary cell types, implying that further (possibly restrictive) mechanisms must exist to account for the final somatotrophic or lactotrophic phenotype.

The nature of such restrictive mechanisms remains unknown, but some further information has recently been gained by the use of transgenic mice. Either the proximal or the distal enhancer elements alone were able to direct a low level of pituitary-specific expression of reporter genes; the two elements together interacted synergistically to give high levels of expression which was almost exclusively restricted to lactotrophs (Crenshaw *et al.* 1989). However, these studies also showed that when the distal enhancer element was isolated from its flanking DNA it directed reporter gene expression to thyrotrophs as well as lactotrophs. Thus a model was proposed in which prolactin gene expression was activated in lactotrophs and restricted in somatotrophs by the proximal and distal enhancer regions, and restricted in thyrotrophs by elements which flank the distal enhancer region (Crenshaw *et al.* 1989). The precise mechanisms involved have yet to be elucidated, but are likely to be a useful model for the study of differentiation in other endocrine and non-endocrine tissues.

### Human prolactin gene

The human prolactin gene contains a long upstream regulatory element which is related to the rat gene, but

much less is known about its regulation. DNase I footprinting studies reveal the presence of three pituitary-specific protein binding sites in the proximal 217 bp of the human prolactin promoter, and two such sites within the human growth hormone and human chorionic somatomammotrophin (CS) genes (Lemaigre, Peers, Lafontaine *et al.* 1989). Competition studies suggested that a single factor bound to all three genes, and that it was related to Pit-1/GHF-1, although the relevant factor has yet to be purified or cloned from human tissue.

One important difference between rat and man is the fact that the human gene is expressed in the decidualized endometrial tissue of the placenta as well as the pituitary (Clements, Whitfield, Cooke *et al.* 1983). The mRNA has a slightly different size (Gellersen, DiMattia, Friesen & Bohnet, 1989), which may suggest a different transcription initiation site and possibly that different transcription factors may be involved. It remains to be seen whether a factor related to Pit-1/GHF-1 is expressed in the human placenta, but the cellular localization of the prolactin and CS genes within the placenta differs, and presumably other factors interact to dictate the ultimate pattern of expression.

### Summary

The past 2 years have seen dramatic progress in our understanding of the molecular basis of prolactin gene expression in the pituitary gland. The identification of the pituitary-specific factor Pit-1/GHF-1 has been a major advance, and it leads to new questions, such as the basis for the tissue-specificity of Pit-1/GHF-1 expression itself. It clearly cannot be the only factor involved, as it is found in three pituitary cell types, yet prolactin is expressed in only one of these. It is probable that a network of *trans*-acting factors, some tissue-specific and some ubiquitous but hormonally activated, interact to determine the overall pattern of expression of the prolactin gene, and the dissection of this system should provide some valuable insights into endocrine gene regulation in general.

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# Regulation of Growth Hormone Secretion and Messenger Ribonucleic Acid Accumulation in Human Somatotropinoma Cells *in Vitro*\*

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**ABSTRACT.** GH secretion and mRNA levels were measured in cultured cells obtained from six human pituitary somatotroph tumors to investigate their hormonal and intracellular regulation. The responses were variable between tumors, but, in general, mRNA levels were less responsive than GH release to *in vitro* manipulation. GH-releasing factor [GRF-(1-29) amide; 10 nM] increased GH release and mRNA levels in three of four tumors tested to 30-97% above control values, but the fourth tumor was unresponsive. Somatostatin (1  $\mu$ M) inhibited GH release significantly in four of the six cases, to 35-79% of control levels, but had no inhibitory effect on GH mRNA accumulation, in contrast to earlier studies on rat pituitary tissue. Bromocriptine (100 nM) likewise inhibited GH release (50-75% of control), but not GH mRNA levels, in the four tumors tested. Forskolin

(10  $\mu$ M; used to activate adenylate cyclase) stimulated GH release and mRNA levels in the two cases that responded most clearly to GRF, but had no significant effect in the other tumors; however, the phorbol ester 12-O-tetradecanoyl phorbol-13-acetate (100 nM) had no consistent effect on mRNA levels despite stimulating secretion in four of six cases. Thus, there was considerable variation in responses among the tumors tested; however, the responsiveness to GRF was approximately paralleled by that to forskolin, consistent with the suggestion that adenylate cyclase activity and responsiveness are variable among these tumors. Furthermore, the divergent effects of somatostatin on GH release and mRNA suggest uncoupling between its receptor and transcriptional regulatory mechanisms. (*J Clin Endocrinol Metab* 69: 704, 1989)

GH SECRETION is regulated *in vivo* primarily by GH-releasing factor (GRF) and somatostatin, although a number of other hormones are involved, including thyroid hormones, glucocorticoids, and insulin. Knowledge of the hormonal control of GH gene expression has so far been largely based on studies of rat pituitary cells (1-3), and the availability of clonal rat pituitary cell lines has allowed detailed examination of the mechanisms involved (4, 5). Detailed study of the endogenous human GH (hGH) gene has been limited by the difficulty in obtaining sufficient tissue, but may be important in relation to the possible pathogenesis of GH-secreting pituitary tumors. Cytoplasmic levels of hGH mRNA in pituitary tumor cells may be suppressed *in vitro* by insulin-like growth factor-I (6) and increased by dexamethasone, but not by thyroid hormone, in contrast to rat GH (rGH) mRNA (7). However, there is so far no information on the possible effects of the other important regulators of somatotroph function on endogenous hGH

gene expression in human pituitary tissue.

In a preliminary report (8) we have presented results of studies on the regulation of PRL and hGH mRNA accumulation in two human pituitary tumors, one prolactinoma, and one somatotropinoma. We now present data on the hormonal and intracellular control of hGH release and mRNA accumulation in cultured cells from six human somatotrophic tumors. We have examined the effects of a GRF fragment [GRF-(1-29) amide], somatostatin, bromocriptine, forskolin, and phorbol ester in order to determine the possible involvement of adenylate cyclase and protein kinase-C in hGH synthesis and secretion.

## Materials and Methods

Pituitary adenoma tissue was obtained for cell culture from routine transsphenoidal or transfrontal hypophysectomy operations on six patients with acromegaly; patient details are given in Table 1. At operation, pituitary tumor tissue was immediately placed in ice-cold culture fluid, which consisted of Dulbecco's Modified Eagle's Medium (Gibco BRL, Paisley, Scotland) supplemented with 10% horse serum and 2.5% fetal calf serum (Gibco BRL) with antibiotics. The tissue was chopped into small fragments and enzymatically dispersed using collagenase and hyaluronidase, as previously described for

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rat pituitary tissue (9). Each pituitary tumor yielded between  $2.0\text{--}7.8 \times 10^6$  cells, with a viability determined by trypan blue exclusion of 84–95%. Dispersed adenoma cells were plated onto plastic culture dishes at a density of  $1\text{--}2 \times 10^5$  cells/well in 2 mL culture fluid, incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>–95% air, and allowed to grow in monolayer culture for at least 5 days before experiments were carried out. The cells were then washed by repeated (serum-free) medium exchanges and treated for 24 h with test substances in serum-free culture fluid. After treatment periods culture fluid was removed for RIA of released hGH, and cell cytoplasmic extracts were prepared for mRNA determinations.

#### mRNA measurements

mRNA accumulation was measured by cytoplasmic dot hybridization using the technique of White and Bancroft (10). Cells were scraped from culture dishes, and cytoplasmic extracts were prepared as described previously (11). Cytoplasmic samples were applied in duplicate to a hybridization transfer membrane, and mRNAs were immobilized by baking before prehybridization, hybridization, and autoradiography (11). A cDNA probe specific for hGH (12) was radiolabeled with [<sup>32</sup>P] dCTP using the oligo labeling technique (13) (Amersham International PLC, Radiochemical Centre, Amersham, United Kingdom) to a specific activity of approximately  $0.5 \times 10^9$  dpm/ $\mu$ g DNA.

The degree of hybridization was assessed by scanning densitometry of developed autoradiographs (11); for each experiment mRNA results were expressed in arbitrary units of optical density relative to control samples, which were assigned the value of 1.0 U (100%). For Northern blot analysis mRNA species were extracted from cytoplasm of cultured cells from one of the tumors, and then separated by agarose gel electrophoresis before hybridization.

#### Materials

A GRF fragment [GRF-(1–29) amide; equipotent to authentic human GRF; Bachem, Saffron Walden, Essex, United Kingdom] and somatostatin-(1–14) (Sigma Chemical Co., Poole, Dorset, United Kingdom) were dissolved directly into culture fluid. Forskolin (Calbiochem, Cambridge, United Kingdom) and bromocriptine (Sigma) were dissolved initially in ethanol, and the phorbol esters 12-*O*-tetradecanoyl phorbol-13-acetate (TPA; Sigma) and phorbol 12,13-didecanoate (PDD; Sigma)

were dissolved in dimethylsulfoxide before dilution in culture fluid immediately before experiments.

#### Hormone assays and statistics

hGH released into culture fluid was measured by RIA, using MRC 66/217 hGH standard. Blank samples consisting of culture fluid alone did not cross-react in this assay. Midrange intra- and interassay coefficients of variation were less than 10%. Results are presented as the mean  $\pm$  SEM, and statistical significance was assessed by one-way analysis of variance.

#### Results

Similar densities of viable cells were plated onto culture wells in each experiment, and the amounts of hGH released by unstimulated tumor cells in culture were similar, except for tumors 3 and 4 where basal hGH release was over 4-fold higher (Table 1).

Cultured cells obtained from tumor 1 were used to prepare RNA for Northern blot analysis (Fig. 1). This confirmed that a single species of hGH mRNA was present, and that its size was approximately 1000 bases, the expected size for this mRNA (14) and in accordance

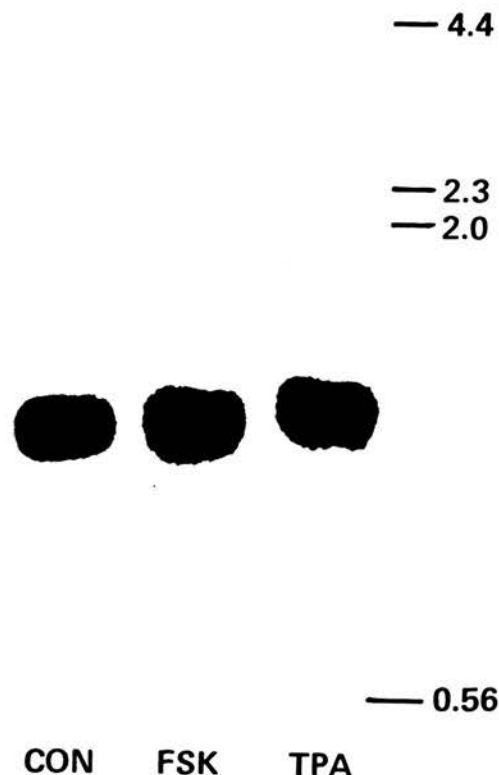


FIG. 1. Northern blot hybridization analysis of RNA (10  $\mu$ g/lane) extracted from cultured cells from tumor 1. The cells were treated for 24 h with control medium (CON), 10  $\mu$ M forskolin (FSK), or 100 nM TPA before extraction of RNA. The RNA was electrophoresed on a 1% agarose gel and blotted onto a hybridization membrane for hybridization with the <sup>32</sup>P-labeled hGH cDNA probe, followed by autoradiography. Standard DNA size markers (in kilobases) migrated as indicated at the right of the autoradiograph. (See also Table 2 for quantitative data for forskolin and TPA from dot hybridization studies.)

TABLE 1. Summary of patient details, showing random preoperative serum GH levels, with mean unstimulated GH release *in vitro* over a 24-h incubation period

Tumor no.	Patient's sex, age (yr)	Basal serum GH preoperatively (mU/L)	Unstimulated GH release <i>in vitro</i> (mU/L)
1	M, 34	4100	75 $\pm$ 10 <sup>a</sup>
2	M, 42	32	67 $\pm$ 10
3	M, 42	160	249 $\pm$ 39
4	M, 39	130	398 $\pm$ 30
5	F, 42	130	64 $\pm$ 4.7
6	F, 36	14	55 $\pm$ 2.5

<sup>a</sup> Mean  $\pm$  SEM.

with one previous report of such studies on human pituitary tissue (6).

The effects of 10 nM GRF-(1-29) amide were studied in four tumors (no. 1-4; Fig. 2); hGH release was stimulated in three of the four tumors, although the degree of stimulation varied from 129-199% of control values. Moderate stimulation of hGH mRNA levels was likewise seen only in these three tumors, with values of 130-167% of the control levels in each case.

Somatostatin (1  $\mu$ M) was studied in all six tumor cultures (Fig. 3). GH release was suppressed in four cases, although the degree of inhibition in the responsive tumors varied from 35-79% of the control values. In contrast, hGH mRNA accumulation was not consistently affected; slight and nonsignificant reductions were seen in three of the six cases, the maximum suppression was only to 70% of control values, and other values ranged between 80-176% of control ( $P = NS$ ).

Bromocriptine (100 nM) was studied in four tumor cultures (no. 1-4; Fig. 4). GH release was reduced in all four cases to between 50-75% of control values (although significantly so in only two), whereas hGH mRNA was

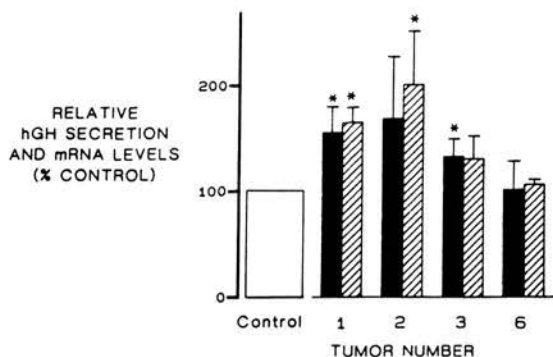


FIG. 2. Effects of GRF on tumors 1, 2, 3, and 6. Cultured pituitary tumor cells were incubated with control medium or 10 nM GRF-(1-29) amide for 24 h. Hormone release data (▨) are expressed in terms of percentage of unstimulated hGH secretion (see Table 1); mRNA data (■) are expressed in arbitrary units of optical density, also as percentage of control values in unstimulated groups of cells. Data are the mean  $\pm$  SEM ( $n = 5-8$  replicate culture wells/group). \*,  $P < 0.02$  compared to control samples for each tumor.

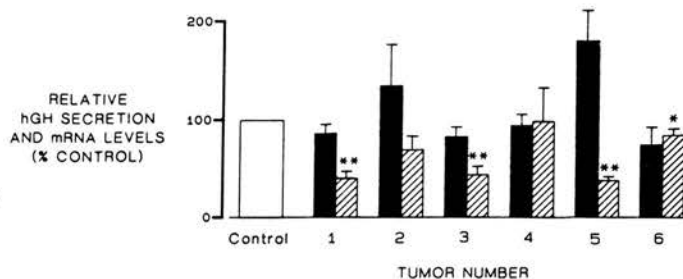


FIG. 3. Effects of somatostatin on tumors 1-6. Cultured pituitary tumor cells were incubated with control medium or 1  $\mu$ M somatostatin for 24 h. Hormone release data (▨) and mRNA data (■) are expressed as in Fig. 2 and are the mean  $\pm$  SEM ( $n = 4-8$  replicate culture wells/group). \*,  $P < 0.02$ ; \*\*,  $P < 0.005$  (compared to control samples for each tumor).

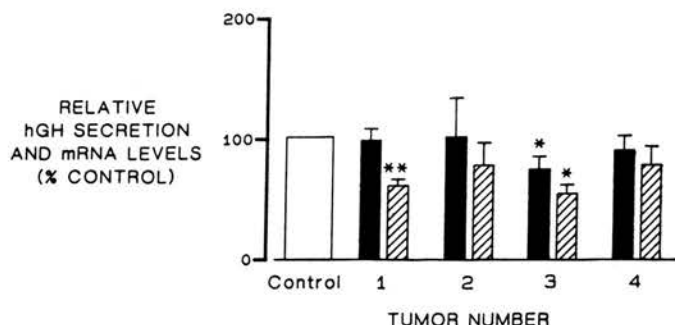


FIG. 4. Effects of bromocriptine on tumors 1-4. Cultured pituitary tumor cells were incubated with control medium or 100 nM bromocriptine for 24 h. Hormone release data (▨) and mRNA data (■) are expressed as in Fig. 2 and are the mean  $\pm$  SEM ( $n = 4-7$  replicate culture wells/group). \*,  $P < 0.05$ ; \*\*,  $P < 0.005$  (compared to control samples for each tumor).

significantly suppressed in only one of the four tumors (to 70% of control) and was unaffected in the others.

All six tumor cultures were treated with 10  $\mu$ M forskolin (to activate adenylate cyclase and, thus, raise intracellular concentrations of cAMP) and the phorbol ester TPA (100 nM; activating protein kinase-C); the results are shown in Table 2 (and see also Fig. 1). Forskolin doubled hGH release and moderately increased mRNA levels in tumors 1 and 2, but had no effect in the others. However, it is noteworthy that tumors 1 and 2 were also those that responded most clearly to GRF, whereas tumor 3 showed a smaller response, and tumor 6 failed to respond to either agent. The tumor-promoting phorbol ester TPA stimulated hGH release in four of the six cases. [In a single experiment tumor 3 was treated with the nontumor-promoting phorbol ester PDD (100 nM), which had no significant effect: hGH release was  $121 \pm 6\%$  of control ( $n = 4$  replicates;  $P = NS$  compared to control) compared to  $193 \pm 25\%$  of control with 100 nM TPA ( $n = 4$ ;  $P < 0.02$ ).] In contrast to hGH release, hGH mRNA levels showed no consistent changes, with nonsignificant stimulation by TPA only in tumors 2 and 6 (Table 2).

## Discussion

A major limitation of studies of human pituitary tissue is the lack of large amounts of tissue or clonal cell lines for *in vitro* manipulation. Cytoplasmic dot hybridization assays have proved valuable in studying mRNA regulation in small tissue samples (10) and were, therefore, used here to investigate hGH mRNA regulation in human pituitary tumor tissue. The present results show 1) that responses of both hGH release and mRNA accumulation are variable between different tumors, but differ from those in normal rat pituitary tissue *in vitro*; 2) that mRNA levels do not necessarily parallel changes in hormone secretion; and 3) that adenylate cyclase and protein kinase-C differentially regulate hGH secretion and mRNA accumulation.



TABLE 2. GH release and mRNA levels in tumors 1-6 in responses to treatment for 24 h with forskolin or phorbol ester (TPA)

	Tumor no.					
	1	2	3	4	5	6
Forskolin (10 $\mu$ M)						
hGH release (% of control)	197 $\pm$ 37 <sup>a</sup>	193 $\pm$ 24 <sup>a</sup>	148 $\pm$ 18	77 $\pm$ 37	82 $\pm$ 5	108 $\pm$ 11
hGH mRNA (% of control)	178 $\pm$ 9 <sup>a</sup>	132 $\pm$ 30	80 $\pm$ 10	93 $\pm$ 44	120 $\pm$ 20	86 $\pm$ 11
TPA (100 nM)						
hGH release (% of control)	426 $\pm$ 70 <sup>b</sup>	263 $\pm$ 39 <sup>b</sup>	193 $\pm$ 25 <sup>a</sup>	55 $\pm$ 8 <sup>a</sup>	120 $\pm$ 6	130 $\pm$ 2 <sup>a</sup>
hGH mRNA (% of control)	109 $\pm$ 20	155 $\pm$ 25	90 $\pm$ 10	80 $\pm$ 30	160 $\pm$ 30	112 $\pm$ 16

Hormone release data are expressed in terms of percentage of unstimulated hGH secretion (see Table 1); mRNA data are expressed in arbitrary units of optical density and in terms of percentage of control values in unstimulated groups of cells (100%). Data are the mean  $\pm$  SEM (n = 3-6 replicate culture wells/group). Significance is indicated compared to control samples for each tumor; all other data are not significantly different from control values.

<sup>a</sup>  $P < 0.02$ .

<sup>b</sup>  $P < 0.001$ .

GH release and mRNA levels were stimulated in three of four tumors by GRF-(1-29) amide. Previous reports have indicated stimulation of hGH release by equipotent doses of GRF-(1-40) and GRF-(1-44) in five of six tumor cultures, although the increases varied between 30-500% above the control value (15). GRF is known to stimulate hGH mRNA levels (2, 3, 16), and one previous report describes stimulation of hGH mRNA accumulation by GRF, although only to approximately 35% above control values (6). Relatively small effects of GRF-(1-29) amide were also seen in the present studies, although this could be partly explained by the absence in the culture fluid of supplementary glucocorticoid, which is known to augment somatotroph responsiveness to GRF (17, 18), or to the relatively short (24-h) incubation period. One tumor was completely unresponsive in terms of both hGH mRNA levels and secretion.

Somatostatin and bromocriptine in high doses had clear inhibitory effects on hGH release, confirming the data of Lamberts *et al.* (19); although mRNA levels were suppressed by bromocriptine in one tumor, no consistent pattern was found for hGH mRNA accumulation in the others, and somatostatin had no effect. The results obtained with bromocriptine accord with previous data from normal rat pituitary tissue (20). There is little information regarding the effects of somatostatin on GH gene expression; using identical experimental techniques and design, Wood *et al.* (20) found that somatostatin treatment (in the presence of dexamethasone) for 24 h reduced rGH mRNA levels to 35% of control values, comparable to the suppression of rGH release (20). However, Gick and Bancroft (21) found no effect of somatostatin on rGH mRNA accumulation in GH<sub>3</sub> cells, while Barinaga *et al.* (16) found no effect on rGH gene transcriptional rate. One previous report, using a quantitative *in situ* hybridization technique to study three human somatotroph tumors, has described the failure of somatostatin to suppress hGH mRNA levels (22). The lack of effect of somatostatin in the present six tumors

cannot be explained by lack of membrane receptors in view of the inhibition of hGH release and might suggest abnormal postreceptor signal transduction.

cAMP is known to be involved in GRF action, and high intracellular levels of cAMP stimulate rGH release and endogenous rGH gene transcription in normal rat pituitary tissue (16) and also the transcription of a reporter gene from the hGH promoter region in transfected rat pituitary cells (23). Forskolin caused significant stimulation of hGH release and mRNA accumulation in only two tumors. These two tumors also showed the most marked responses to GRF-(1-29) amide, implying that the coupling between cAMP generation and hGH gene transcription and hormone release was intact in these cases; on the other hand, the lack of response to either GRF or forskolin in the other tumors might suggest uncoupling at some level between receptor and cellular response. Vallar *et al.* (24) found a subgroup of about 30% of somatotroph tumors characterized by higher hGH secretion rates and high intracellular cAMP levels; in these tumors adenylate cyclase activity was not stimulated by GRF, and this was related to the presence of an altered G<sub>s</sub> protein. In the absence of data for cAMP accumulation it is not possible to say whether the present results indicate two differing types of tumors with distinctive levels of basal adenylate cyclase activity, although there was no correlation between GRF-responsiveness and the absolute level of basal hGH secretion.

In previous studies of rat pituitary tissue, activation of protein kinase-C by phorbol esters was found to stimulate rGH release without increasing rGH gene transcription, in contrast to activation of adenylate cyclase, which increased both (16, 25). In the present study TPA was able to stimulate hGH release up to 4-fold basal levels, demonstrating that protein kinase-C can mediate hGH release. However TPA had no consistent effects on hGH mRNA levels, suggesting that, as in the rat, protein kinase-C has no effect on hGH gene regulation.

In conclusion, the present data show that the previ-

ously recognized heterogeneity in the patterns of hGH release by pituitary tumor cells *in vitro* also applies to hGH mRNA accumulation. However, more importantly, the data imply that there may be divergences between the regulation of hGH secretion and mRNA production, as in the case of somatostatin, which could result in the rate of gene transcription being relatively autonomous and independent of receptor-mediated inhibitory control. Uncoupling between cAMP production and hGH production may be a further factor in a group of somatotrophic tumors. Several different mechanisms may underlie the development of adenomatous change in the pituitary gland, including hypomethylation of the hGH gene itself (26), amplified proto-oncogene expression (26), and abnormal signal transduction (24). The apparent relative autonomy of hGH gene transcription seen in the present study may be related to these findings and could be involved in tumor pathogenesis.

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# Calcium and Calmodulin Regulation of Prolactin Gene Expression

Julian R. E. Davis

*Prolactin gene transcription is critically dependent upon intracellular calcium and the calcium-binding protein calmodulin. The effects of calcium and calmodulin on the prolactin gene appear to involve defined regions of the 5'-flanking sequence, and interactions between nuclear proteins and this DNA may be related to mechanisms of transcriptional control by pituitary cell-type-specific trans-acting factors.*

Intracellular calcium is an important regulator of a variety of cellular functions, and it is involved in the secretion and synthesis of many different hormones. The cytoplasmic concentrations of calcium are affected by hormonally induced influx of extracellular calcium ions, and calcium mobilization from intracellular stores, probably the endoplasmic reticulum. In many cell types the resultant intracellular concentrations of free calcium are not stable but oscillate, and some calcium-mediated responses may in fact be frequency-modulated rather than amplitude-modulated (Schlegel et al. 1987). The precise mechanisms for calcium action within the cell are not yet known; it may exert direct effects, or it may act through one or more calcium-binding proteins or kinases, for example calmodulin and protein kinase C.

Prolactin secretion by pituitary lactotrophic cells is stimulated by thyrotropin-releasing hormone (TRH)-induced hydrolysis of phosphatidylinositol bisphosphate, which is thought to lead to a rapid mobilization of intracellular calcium stores, with a subsequent slower influx of extracellular calcium (Gershengorn 1986). Whereas the acute release of prolactin by TRH is now well recognized to be calcium dependent, there is increasing evidence for a crucial role of calcium in the regulation of pro-

lactin gene transcription, and some clues as to the possible mechanisms involved are emerging from recent work.

## • Regulation of the Prolactin Gene

Prolactin gene transcription (and mRNA accumulation) is influenced by a variety of hormones, including estrogen, thyroid hormone, and glucocorticoids, acting through nuclear receptors; and dopamine, TRH, vasoactive intestinal peptide, and epidermal growth factor (EGF), acting through membrane receptors coupled to a variety of intracellular second messenger systems (Davis et al. 1989). Recently, the general actions of a number of intracellular regulators have been studied, and important advances have been made in our understanding of the mechanism of action of cAMP and protein kinase C. Much less is yet known about calcium and the calcium-binding protein calmodulin, although the calcium-calmodulin system appears to be important in regulating prolactin gene expression. A particular puzzle in the case of the prolactin gene is the very wide range of known regulators and intracellular signals that affect its expression; are the mechanisms different in each case, or do they converge onto a small number of common targets at the level of the gene itself?

## • Effects of Calcium

Evidence for a role for calcium in prolactin gene regulation has come from a va-

riety of experimental approaches. However, it remains controversial whether calcium is a major regulator in its own right, or is simply an essential cofactor. Calcium entry into cells may in certain circumstances stimulate prolactin gene transcription with a resultant increase in mRNA levels. White et al. (1981) deprived clonal GH<sub>3</sub> cells of extracellular calcium and then added calcium chloride to the culture fluid, which resulted in over fivefold stimulation of prolactin mRNA levels. Similar experiments in normal rat anterior pituitary cells showed that these effects of calcium were much greater for prolactin than for growth hormone (Gick and Bancroft 1985) and were reversible on withdrawal of calcium. Removal of calcium from extracellular culture fluid by EGTA rapidly reduced accumulation of prolactin mRNA to undetectable levels, whereas growth hormone mRNA was much less affected (Gick and Bancroft, 1985). Under these conditions TRH action was abolished, although EGF still induced some increase in mRNA levels (White and Bancroft 1983). Hence, expression of the prolactin gene is reduced in low-calcium conditions, and increases on restoration of normal intracellular calcium. But while these effects are greater for prolactin than for growth hormone, they do not necessarily imply that calcium per se is a major regulator of prolactin gene expression in calcium-replete cells.

Pharmacological manipulations of calcium flux have involved ionophores or calcium channel agonists or antagonists, but have given conflicting results. Murdoch et al. (1985) found that neither of the calcium ionophores ionomycin and A23187 had any effect on the rate of gene transcription in GH<sub>4</sub> cells, implying that elevation of intracellular free calcium was not a sufficient stimulus. However, the ionophores did augment the stimulation achieved with phorbol ester to levels comparable with those induced by TRH, suggesting that elevation of intracellular calcium was acting as a cofactor in concert with protein kinase C, but was inactive alone. In contrast to these results, Laverrière et al. (1988) found that both ionomycin and A23187 appreciably enhanced transcription in GH<sub>3</sub>B6 cells, implying that mobilization of intracellular calcium by itself was in fact a sufficient stimulus. In addition, influx of extracellular calcium

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through voltage-sensitive channels induced by the dihydropyridine calcium channel agonist Bay K 8644 increases transcriptional rate and mRNA levels (in cells never deprived of calcium), similar in magnitude to the effect of TRH but not additive to the TRH effect (Laverrière et al. 1988; Hinkle et al. 1988). The differences between ionophores and channel agonists may be explained by the disruptive effects of ionophores on intracellular calcium gradient and pools (White et al. 1989).

The importance of extracellular calcium has been studied with the use of various calcium channel blocking agents, including verapamil and nifedipine; these agents slightly reduce intracellular free calcium concentrations, but markedly reduce prolactin gene transcription and cytoplasmic mRNA accumulation. They do not, however, prevent the severalfold stimulation induced by TRH (Murdoch et al. 1985; Davis et al. 1988; Hinkle et al. 1988), implying that a stimulatory effect of TRH may be at least partly independent of calcium influx.

Regarding the role of intracellular calcium, pure intracellular antagonists of calcium action have not been identified, but several divalent cations, including manganese and cobalt, are thought to have intracellular as well as channel-blocking effects. Both these cations dramatically reduce prolactin secretion, and cobalt chloride has been shown to inhibit prolactin gene transcription and reduce mRNA levels, also abolishing the

these data have to be interpreted with some caution.

Transcription of a number of other genes is now known to be calcium-responsive, indicating that the marked calcium responsiveness of the prolactin gene is not unique. These include the glucose-regulated genes p3C5 and p4A3, related to the heat shock protein family (Resendez et al. 1986), and also the cellular proto-oncogene *c-fos* (Bravo et al. 1985; Morgan and Curran 1986), which in pituitary cells is induced relatively slowly by calcium, in parallel with the prolactin gene (Bandyopadhyay and Bancroft 1989). Indeed, a calcium-responsive element has been demonstrated for this gene (Sheng et al. 1988), which also confers responsiveness to cAMP (Sassone-Corsi et al. 1988).

In summary, there is controversy over the precise function of calcium in the regulation of prolactin gene expression. Calcium is clearly a cofactor for protein kinase C action; however, calcium may in some circumstances be a sufficient stimulus in its own right to increase prolactin gene transcription and mRNA levels, and this effect depends on both intracellular and extracellular calcium.

#### • Mechanisms for Calcium Action

The precise function of calcium in prolactin gene regulation is controversial, but evidence exists for a potential role for calcium as a regulator in its own right. The mechanism of calcium action on the prolactin gene may involve the

the calcium induction of the prolactin gene (Bandyopadhyay and Bancroft 1989), suggesting that this kinase is not an essential intermediate in this action of calcium.

Studies of the role of calmodulin have depended heavily on the use of calmodulin antagonists such as the phenothiazine drugs trifluoperazine and calmodazolium. These reduce both gene transcription rate and prolactin mRNA accumulation (Bancroft et al. 1985; White 1985; Murdoch et al. 1985). Their specificity is, however, relatively poor as they also inhibit a number of other intracellular enzymes, including protein kinase C. The more recently introduced naphthalene sulfonamide derivatives such as W7 are much more specific antagonists, and, in fact, these drugs have similar dramatic effects in reducing both basal and TRH-stimulated prolactin RNA accumulation, with relatively small effects on growth hormone mRNA levels (Davis and Wilson 1989).

#### • DNA Elements

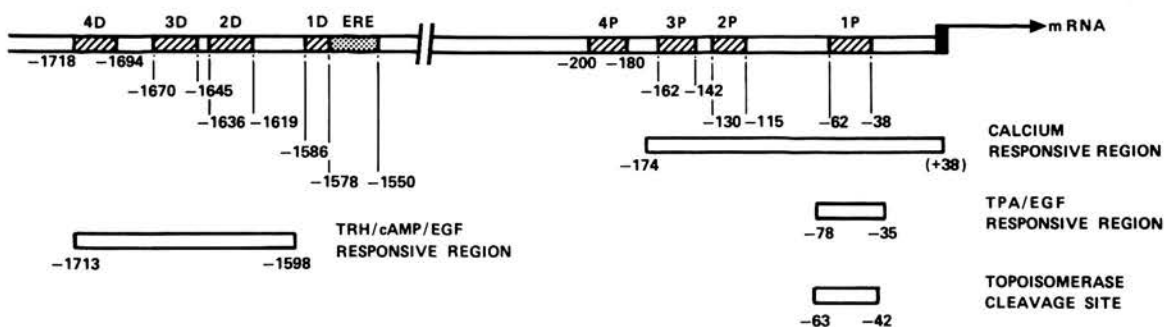
A major advance in our understanding of prolactin gene regulation has been the identification of pituitary-specific nuclear protein interactions with the upstream regulatory 5'-flanking DNA. The prolactin gene is now known to contain a long upstream regulatory region (Figure 1), which consists of a proximal enhancer region (up to 400 bp upstream) and a distal region (up to 1700 bp upstream) (Nelson et al. 1986). Both of these regions are important in maintaining transcriptional activity, although their relative importance is disputed. The proximal region contains four sites (1P-4P) that bind a pituitary-specific protein variously termed "Pit-1," "PUF1," or "LSF-1" (Gutierrez-Hartmann et al. 1987; Cao et al. 1988; Nelson et al. 1988; Lufkin et al. 1989); the distal region is reported to contain up to four "Pit-1" sites (1D-4D; Nelson et al. 1988), one of which is close to an estrogen receptor binding region (Kim et al. 1988).

Response elements have recently been defined for some intracellular regulators. For example, the transcriptional response to both EGF and protein kinase C activation by phorbol ester is conferred by DNA sequences between positions -35 and -78 bp upstream from the cap site (Elsholtz et al. 1986), and this coincides with the first proximal

*IT REMAINS CONTROVERSIAL WHETHER CALCIUM IS A MAJOR REGULATOR IN ITS OWN RIGHT, OR IS SIMPLY AN ESSENTIAL COFACTOR.*

stimulation normally seen with TRH (Murdoch et al. 1985; Davis et al. 1988). These effects were much greater than those seen with high doses of calcium channel blockers, suggesting that calcium influx into these cells was less critical than intracellular calcium. These agents are, however, quite markedly cytotoxic at high concentrations, and thus

calcium-dependent kinase protein kinase C and calmodulin. Activation of protein kinase C by the phorbol ester TPA stimulates prolactin gene transcription (Murdoch et al. 1985), and this kinase requires calcium as a cofactor (Nishizuka, 1984). Down-regulation of protein kinase C by chronic exposure to phorbol esters, however, does not affect



**Figure 1.** Schematic representation of the 5'-flanking region of the rat prolactin gene. Approximate boundaries of reported pituitary cell-specific protein binding sites are indicated, with putative response regions indicated below (for references, see the text). ERE, estrogen response element; and TPA, phorbol ester.

site (1P) for pituitary-specific nuclear protein binding (Nelson et al. 1988). A precise response element for calcium has not yet been identified, but the information required for a full response to calcium (induced in both calcium-deprived cells by calcium and in calcium-replete cells by Bay K 8644) is contained within the first 174 bp of DNA upstream from the cap site (Jackson and Bancroft 1988), and the proximal 395 bp of upstream DNA also confer very marked calmodulin sensitivity onto a reporter gene (Davis and Wilson 1989). Potential response elements may, however, be multiple, as TRH, EGF, and cAMP effects may be conferred on a reporter gene by distal as well as by proximal enhancer regions of the prolactin gene (Day and Maurer 1989), implying interactions between DNA-bound proteins in both proximal and distal enhancer regions. The 174 bp calcium-responsive stretch of the prolactin 5'-flanking DNA contains three of the four proximal "Pit-1" binding sites, inviting the speculation that the binding or efficacy of such a protein may in some way be regulated by calcium itself, or by a separate calcium/calmodulin-dependent protein.

#### • DNA-Binding Protein Factors

One calcium- and calmodulin-dependent protein that has received recent attention is DNA topoisomerase. Phosphorylation of this enzyme by protein kinase C increases its DNA unknotting and relaxing activity, and it has been

implicated in the regulation of some prokaryotic genes (Wang 1985). Specific inhibitors of this enzyme markedly reduce the induction of prolactin mRNA production by calcium, and three major DNA cleavage sites for this enzyme are found in vitro within the 5'-flanking region of the gene (White and Preston 1988). One of these sites was found in a region (-42 to -63 bp) that contained consensus recognition sequences for this enzyme, but which also coincides with the first "Pit-1" site and EGF/phorbol ester response element.

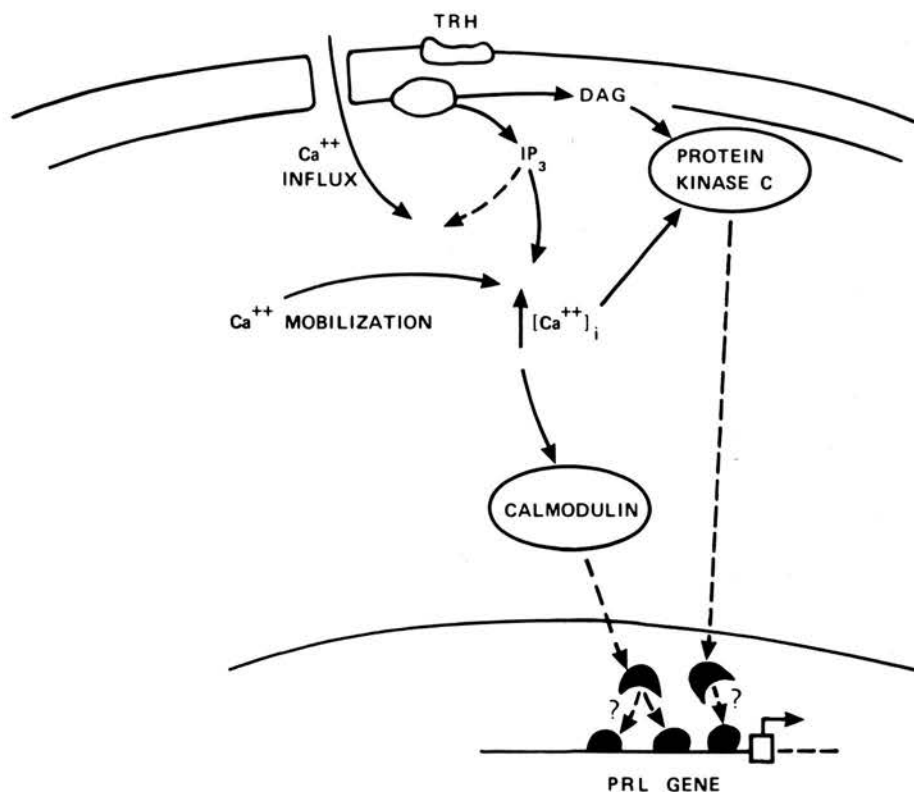
The coincidence of response regions for calcium and EGF/phorbol ester and a potential cleavage site for DNA topoisomerase with pituitary-specific protein binding sites suggests that these intracellular signals may exert their effect by modifying the binding of tissue-specific proteins to DNA or to other DNA-binding factors, or alternatively, by modifying already bound *trans* factors to affect their interaction with the transcription initiation complex. So far there are few data available that address this question. Gel retardation assays have indicated that the pattern of binding of pituitary nuclear proteins to the EGF/phorbol ester response element (site 1P) is unaffected by a 1-h exposure of cells to phorbol esters (Elsholtz et al. 1986). A short exposure of pituitary cells to specific calmodulin antagonists, however, dramatically alters the pattern of protein binding to site 3P (-138/-162) in a similar assay (Davis, unpublished data), suggesting some calmodulin-sen-

sitive protein-DNA interaction, though the proteins involved are as yet uncharacterized.

#### • Conclusions

Regulation of prolactin gene expression involves calcium and calmodulin, but although a lot has been learned about some aspects of this regulation, the precise mechanisms for calcium effects on target DNA sequences have yet to be discovered. Of the possible intermediates in calcium action, calmodulin now appears to be a more likely candidate than protein kinase C, and DNA topoisomerase is one potential substrate for calmodulin that appears to act at appropriate sites in the proximal 5'-flanking region of the gene. The data so far available also hint at likely interactions with the tissue-specific nuclear protein that binds to multiple sites in both proximal and distal enhancer sites upstream of the transcription initiation site, though such mechanisms remain speculative (Figure 2). There may be at least a functional relationship between the prolactin gene and that for *c-fos*. Although *c-fos* has a very general role in cell function, it shares marked calcium-dependence with the prolactin gene (Bandyopadhyay and Bancroft, 1989), and *c-fos* mRNA levels are regulated similarly to prolactin secretion (Gourdji et al. 1989). The kinetics of *c-fos* induction in GH<sub>3</sub> pituitary tumor cells are unusually slow, without the rapid decline in mRNA levels seen in other cell types. Moreover, one report suggests that the *c-fos* gene was amplified in cells from a human prolactin-secreting pituitary tumor (U et al. 1988). A functional link between the two genes remains speculative, but is of particular interest in terms





**Figure 2.** Tentative and simplified model for calcium action in lactotrophic cells: DAG, diacylglycerol; and IP<sub>3</sub> inositol triphosphate. Pituitary cell-specific nuclear proteins are represented as bound to sites 1P-3P in 5'-flanking DNA of the prolactin gene, interacting with putative calmodulin- or kinase C-sensitive nuclear protein(s).

of the pathogenesis of human pituitary adenomas, as it may provide some insight into the close relationship between prolactin production and lactotroph proliferation seen in these tumors.

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## Articles appearing in our next issue

### ENDOCRINE ROUNDS

#### Diabetes and Hypertension

Donald Simonson

### EMERGING TECHNIQUES

#### Imaging Techniques for Parathyroid Tumors

Gary G. Winzelberg

### BRIEF REVIEWS

#### Growth Hormone-Binding Proteins

Gerhard Baumann

#### Modulation of Hormone Response Elements by Promoter Environment

William J. Roesler, Edwards A. Park, Dwight J. Klemm, Jinsong Liu, Austin L. Gurney, G.R. Vandenbark and Richard W. Hanson

#### The Thyroglobulin Gene: Evolutionary and Regulatory Issues

Daniel Christophe and Gilbert Vassart

#### Ontogeny of Pituitary-Gonadal Interactions: Current Advances and Controversies

Ilpo T. Huhtaniemi and Dwight W. Warren

#### Signal Transduction Processes Leading to Acrosomal Exocytosis in Mammalian Spermatozoa

Gregory S. Kopf and Mary W. Wilde

### BOOK REVIEWS

#### Diabetes for History Buffs

Review by Murray Saffran

#### About Males

Review by Stephen J. Winters

#### Unraveling the Mysteries of Thyroid Growth

Review by M. Saeed-uz-Zafar

#### Menopause Revisited

Review by Najib G. Wakim

#### A New Compendium for Pediatric Bone Disease

Review by Thomas Carpenter

#### Both Sides of the Reproductive Coin Examined

Review by Michael P. Diamond

### BOOKS RECEIVED

### CALENDAR

## Pioneering in Pancreatic Transplantation

### International Handbook of Pancreas Transplantation

Edited by J. M. Dubernard and D. E. R. Sutherland, *Developments in Surgery Series. Dordrecht, Kluwer, 1989, \$185.00. (xi + 552 pages). ISBN 0-89838-399-4.*

The contents of the book are aptly reflected in its title. Published in 1989, the authors' bibliographies extend through 1987. Approximately 100 contributors from 14 countries of western Europe and the United States have produced this volume, which is divided into 18 chapters. Commentaries supplement each chapter.

The book is about the treatment of severe diabetes mellitus by pancreatic

allotransplantation. It covers the pathogenesis of diabetes, the history of pancreatic transplantation, and the indications for transplantation of the pancreas alone or in association with renal transplantation.

Techniques, pre- and postoperative care, complications, and results are comprehensively covered. Conclusions are based in part on the results in 1077 recipients of primary pancreatic transplants reported to a central registry by April, 1987. Two-thirds of the patients had received simultaneous renal grafts.

Among the highlights of the volume is a chapter on "Surgical Techniques and Complications" by Dubernard and colleagues and another by Dubernard and

Sutherland, "Perspectives in Pancreatic Transplantation." Two chapters on indications for transplantation are clearly presented.

With so many contributors, some duplication is unavoidable. The book is beautifully produced by the publisher and carefully edited by two leaders in this highly specialized field. This book, authoritative at present, is essential to any surgeon or scientist performing, supporting, or considering pancreatic transplantation. The authors have succeeded in what they set out to do. **TEM**

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## From Seat of the Soul to Timekeeper

### The Pineal: Endocrine and Non-endocrine Function

Sue Binkley, *Englewood Cliffs, New Jersey, Prentice Hall, 1988 (304 pages). ISBN 0-13-0676-189.*

As an early pineal worker, I received great satisfaction from reading Sue Binkley's book on the present status of the pineal as a physiologically important organ and as the focus of research as a model neuroendocrine system. Binkley has summarized and organized research since the 1960s that has clearly established the sensitivity and responsiveness of the pineal gland to environmental light and its role as a biological timekeeper. Binkley also introduces us to other, less defined areas of pineal investigation.

This is one of the first volumes in a series focusing on endocrinology and intended to supplement standard texts. The author, as mentioned in her preface, has written this volume on the pineal gland for senior undergraduate and graduate students; therefore, this volume serves as an entry level text for those unfamiliar with the pineal or endocrinology in general.

The teaching focus is particularly obvious in the second chapter, in which the methods for both in vivo and in vitro study are outlined and explained. The material is clearly at the level of the target group and would serve as a good source book for individuals needing more information on 24-h measurement protocols, pinealectomy, ganglionectomy, preparation of silastic capsules, and pineal enzyme assays, along with the means of measuring melatonin, chemically and by bioassay.

One particular strength of the book is its focus on comparative anatomy and physiology. The author has carefully pointed out the responses common to many species as well as those areas where there are species-specific differences. For example, there are quite different responses between those animals that synthesize melatonin only in the pineal gland (rats) and those that also make comparable amounts in the retina of the eye (chickens). The book is particularly good for descriptions of these differences and their subtle influence on physiological responses. Chapters 5-8 are the strongest in the book. They serve as an excellent introduction to circadian

rhythms, photoperiodism, and responses to varying light/dark. There is a cohesiveness to these matters, with good explanations of pineal function well supported by experimental evidence and clearly presented in graphs and tables.

Other areas of pineal physiology are still unclear and ambiguous—hibernation, coloration, thermoregulation, and interactions with other endocrine systems. Differences may relate to species as indicated by Binkley, but other possible differences, such as the experimental procedures, are not assessed. Some parts of the book, such as chapters 9-12, become litanies of experiments performed. There is some attempt to correlate disparate results but no clear analysis of data. One begins to wonder whether the author is listing every experiment ever performed or if some minimal criteria of validity have been applied. While there is some value to a listing of work in the field as a central source for other investigators, the reader does not have any sense of value or connection. For example, in Chapter 12, which focuses on pineal function in human subjects, the section on reproduc-